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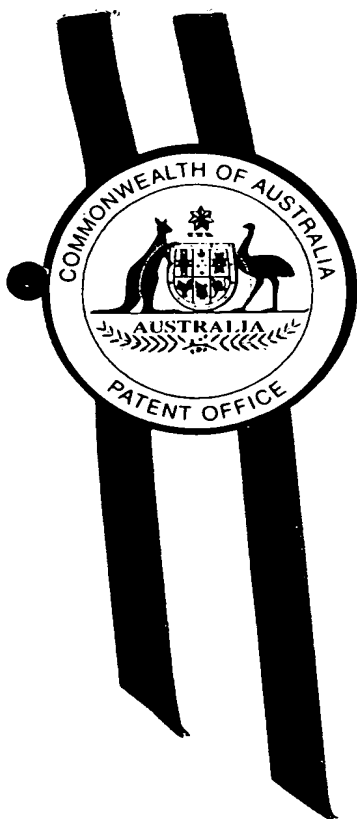
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I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 6164 for a patent by THE UNIVERSITY OF QUEENSLAND filed on 25 September 1998.

WITNESS my hand this
Twenty-eighth day of October 1999

LEANNE MYNOTT
TEAM LEADER EXAMINATION
SUPPORT AND SALES



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PROVISIONAL SPECIFICATION

Applicant(s): THE UNIVERSITY OF QUEENSLAND

Invention Title: SYNTHESIS OF CYCLIC PEPTIDES

The invention is described in the following statement:

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SYNTHESIS OF CYCLIC PEPTIDES

This invention relates to methods for preparing cyclic peptides and peptidomimetics in solution and bound
5 to solid supports, and to cyclic peptide or peptidomimetic
libraries for use in drug screening programmes. In
particular the invention relates to a generic strategy for
synthesis of cyclic peptides or peptidomimetics which
enables the efficient synthesis under mild conditions of a
10 wide variety of desired compounds.

BACKGROUND OF THE INVENTION

Although the development of recombinant DNA
technology and the identification and isolation of proteins
15 mediating a wide variety of biological activities has
enabled the development of new drug therapies, proteins in
general suffer from the disadvantage of susceptibility to
breakdown by digestive and other enzymes. This means not
only that these agents usually have to be administered by
20 injection, but that they also have a short half-life in the
body.

The biological activities of a protein rely on
the three-dimensional structure of the protein molecule,
which results predominantly from a balance between a
25 variety of different non-covalent interactions. In an
attempt to improve the stability and acceptability of
protein pharmaceuticals, both relatively short peptide
sequences encompassing the active site of the protein and
synthetic molecules which adopt a three-dimensional
30 structure resembling the active site have been extensively
investigated. Structurally-constrained peptides in which a
framework is maintained by disulphide bonds as well as by
non-covalent interactions, and cyclic peptide or
peptidomimetic systems in which the cyclisation provides
35 the structural constraint, provide two particularly
attractive approaches to stabilisation of these molecules.

Cyclic peptides show a wide variety of potent biological activities. They have been extensively explored in the drug development process as a means of introducing conformational constraints for the evaluation of the structural, conformational and dynamic properties that are critical to biological activity. Some cyclic peptides are useful as drugs in their own right. Others have been engineered to provide a multitude of functions, including novel biological properties, platforms for the development of protein mimetics, nanotechnology, specific metal coordination sites, and catalysts, to name a few.

Cyclisation may be accomplished by disulfide bond formation between two side chain functional groups, amide or ester bond formation between one side chain functional group and the backbone α -amino or carboxyl function, amide or ester bond formation between two side chain functional groups, or amide bond formation between the backbone α -amino and carboxyl functions.

The potential utility of this class of compound in any application is hindered by difficulties in synthesising the compounds. Whilst the synthesis of the linear precursors generally proceeds in high yield and purity, the final cyclisation reaction can be troublesome, resulting in low yields and/or impure products. This is particularly so for cyclic peptides of fewer than seven amino acid residues, with synthesis of cyclic tetrapeptides resulting in little or no cyclic material.

These cyclisation reactions have been traditionally carried out at high dilution in solution. With the advent of orthogonal protection strategies and new resins for solid phase peptide synthesis, cyclisation has been accomplished while the peptide is attached to the resin. One of the most common ways of synthesising cyclic peptides on a solid support is by attaching the side chain of an amino acid to the resin. Using appropriate orthogonal protection strategies, the C- and N-termini can be selectively deprotected and cyclised on the resin after

chain assembly. This strategy is widely used, and is compatible with either *tert*-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) protocols. However, it is restricted to peptides that contain appropriate side chain
5 functionality to attach to the solid support. It is therefore not amenable to the combinatorial synthesis of arrays of cyclic peptides.

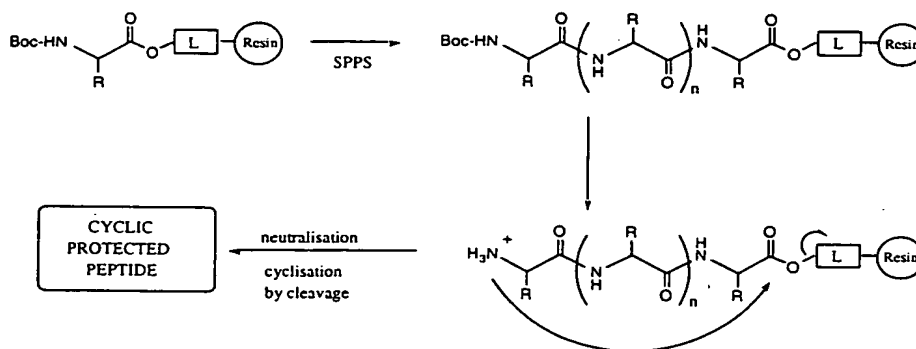
A number of approaches have been used in an attempt to achieve efficient synthesis of cyclic peptides.

10

LINKERS

a) Activated Linkers

One procedure for synthesising cyclic peptides is based on cyclisation with simultaneous cleavage from the
15 resin. After an appropriate peptide sequence is assembled by solid phase synthesis on the resin or a linear sequence is appended to resin, the deprotected amino group can react mildly with its anchoring active linkage to produce protected cyclic peptides, as shown schematically in
20 Scheme 1.



25

Scheme 1

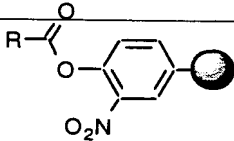
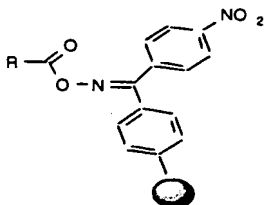
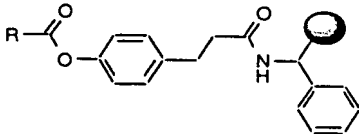
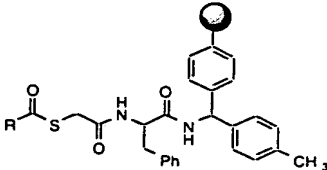
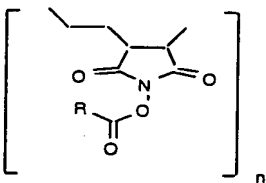
Solid phase cyclic peptide synthesis with activated linkers

Various linkers that have been used for the synthesis of cyclic peptides, or are amenable to their
30 synthesis, are shown in Table 1.

Table 1

Examples of Activated Linkers Amendable
to Solid Phase Cyclic Peptide Synthesis

5

Linker	Reference
	Fridkin et al, 1965; Fridkin et al, 1968
	Osapay and Taylor, 1990; Osapay et al, 1990
	Rivaille et al, 1980
	Richter et al, 1994
	Fridkin et al, 1972; Laufer et al, 1968.

R = Peptide ,  = support

These cleavage-by-cyclisation strategies produce protected cyclic peptides, necessitating a final deprotection step to synthesise the target cyclic material. The cyclisation reaction is generally slow and low in yield, because extended conformational preference of the linear analogue impedes the final cyclisation reaction.

b) Safety Catch Linkers

Extensions of these concepts include supports that can be selectively modified at the end of the assembly to increase the lability of the linker. These linkers are stable during peptide assembly, and are selectively activated, leading to cyclisation and cleavage from the resin. In general, a final deprotection step is required to yield the target cyclic peptide. Examples of linkers that can be used for this approach are shown in Table 2.

Table 2

Examples of Safety Catch Linkers for Solid Phase Peptide Synthesis

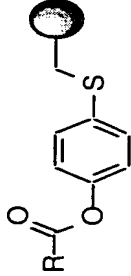
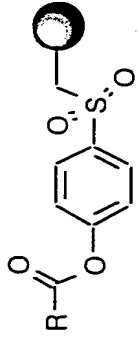
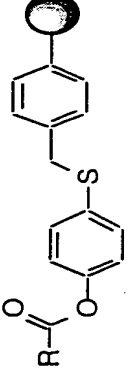
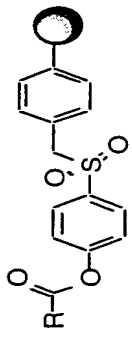
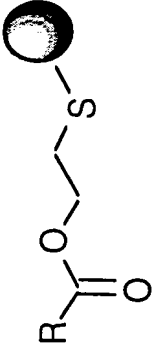
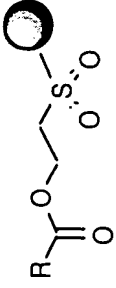
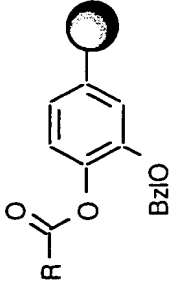
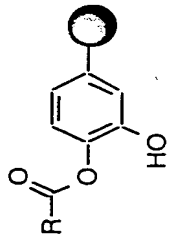
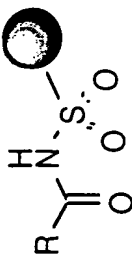
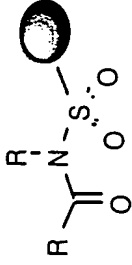
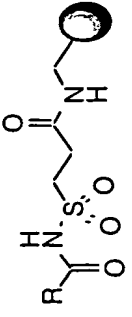
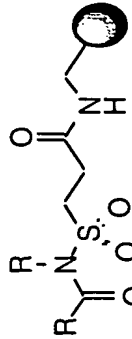
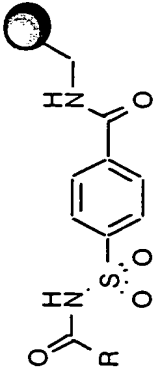
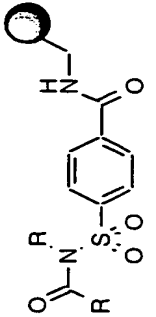
Safety Catch	Reagent	Activated Linker	Ref.
	H ₂ O ₂		Flanigan and Marshall, 1970
	mCPBA/Dioxane		Marshall and Liener, 1970
	H ₂ O ₂		Flanigan, 1971
	HBr		Flanigan, 1971

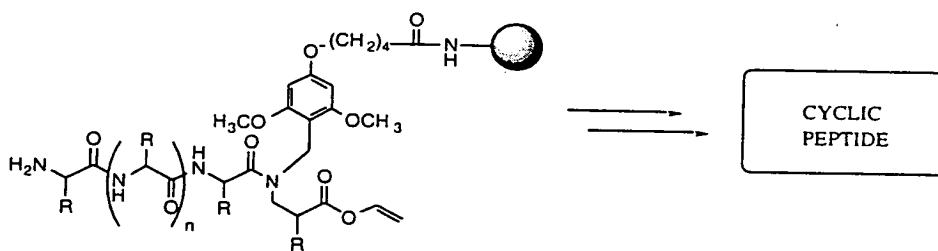
Table 2 (cont.)

	CH ₂ N ₂		Kenner et al, 1971
	ICH ₂ CN		Backes et al, 1996
	CH ₂ N ₂		Backes and Ellman, 1994

These strategies are again limited by the conformational preferences of the linear precursor.

c) Backbone Linkers

5 A simple extension of the concept of attaching the side chain to resin to achieve C- to N-cyclisation is the attachment of the backbone N to resin. Recently Jensen
 10 et al (1996) reported a backbone linker that has been used for synthesising linear peptides, diketopiperazines, peptide aldehydes and cyclic peptides (Jensen et al, 1998). There are several limitations to this process, these include difficulties in acylating the secondary amine to
 15 form the 'linked' amide bond and the fact that standard Fmoc SPPS leads to almost complete diketopiperazine formation at the dipeptide stage. Special protection strategies need to be employed to avoid this problem.



20

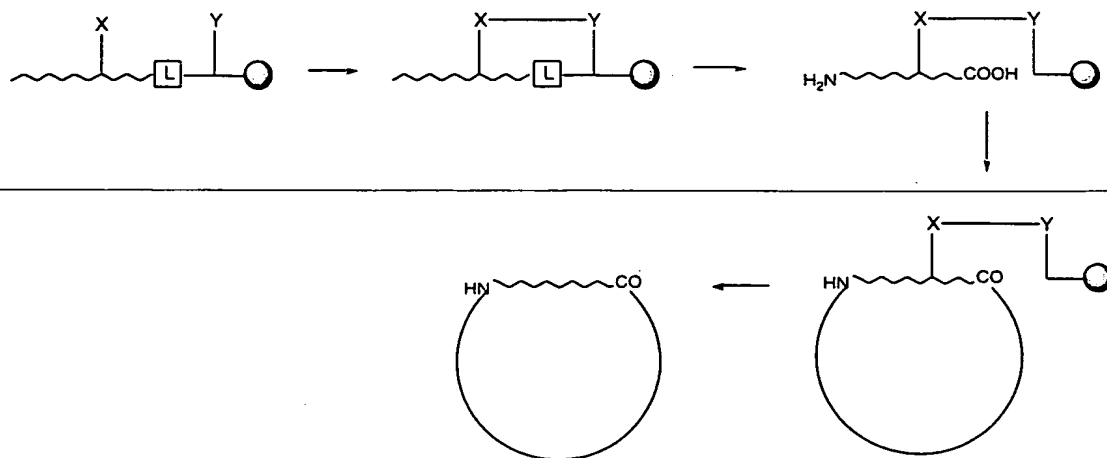
Scheme 2

Backbone linkers for solid phase peptide synthesis

Intraresin Chain Transfer

25 Another approach for synthesising cyclic peptides involves the attachment of a linker that contains two peptide attachment points to the resin, one of which is temporarily masked. Using standard solid phase techniques, the linear precursor is assembled on resin. The X and Y
 30 functionalities (Scheme 3) are then selectively unmasked and cyclised. Cleavage at the linker liberates the free C-terminal carboxylic acid group while the peptide is still attached to the resin. C- and N-cyclisation is then

achieved by standard activation conditions, yielding cyclic peptides.



5

Scheme 3

Linker combination for solid phase peptide synthesis

This method is somewhat limited by the incorporation of the appropriate functionality X into a peptide sequence, and the complex deprotection strategies required. Once again, due to the extended nature of the linear precursors, cyclisation yields would be low.

15 Preorganising Peptides for Cyclisation

a) Reversible N-substitution

The formation of a peptide ring, like any other cyclisation reaction, requires the generation of mutually reactive chain ends, and the reaction of these ends under conditions favouring intramolecular processes. The ease of formation of the ring is related to the conformational stability of the ring and to the losses of internal degrees of freedom that occur upon ring formation. Consequently the presence of turn-inducing amino acids such as Gly, Pro or a D-amino acid enhances the conformational stability of the ring and improves cyclisation yields. For linear peptides that do not contain amino acid residues that stabilise turn structures, the cyclisation reaction is

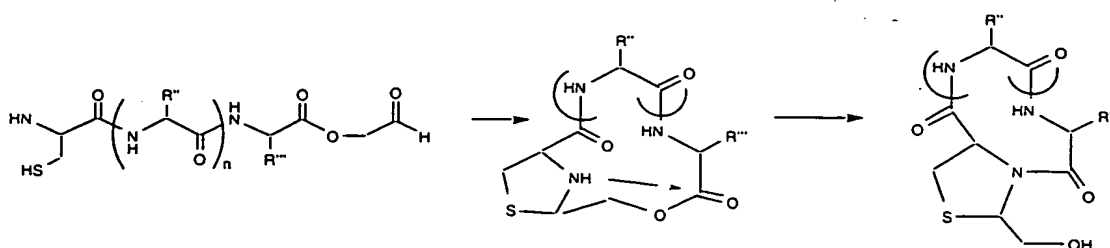
likely to be an inherently improbable or slow process, due to the preference for extended conformations resulting in large strain upon ring formation.

5 This has led to the utilisation of various
reversible chemical modifications of the peptide main
chain, to enhance the *cis* amide bond conformation and hence
reduce ring strain upon cyclisation, and to improve
cyclisation yields. In the synthesis of cyclo-[Phe Phe
Phe Phe], each amide N was substituted with a Boc
10 (Cavelier-Frontin et al, 1993). In this instance the
cyclisation yield increased from less than 1% to 27%.
Similarly, the use of the *N*-(2-hydroxy-4-methoxybenzyl)
(HMB) group as a reversible *N*-substituent has resulted in
similar increases in yields of cyclic peptides (Ehrlich et
15 al, 1996; Ehrlich et al, 1996), although no systematic
study has been undertaken to quantify these effects. From
the point of view of constructing peptide libraries it is
impracticable to substitute every amide N of the linear
precursor.

20

b) Ring Contraction

Ring contraction chemistry can be used for
initial formation of larger flexible rings where the
desired C- and N-termini are appropriately positioned to
25 "snap shut" in a ring contraction reaction to yield the
target cyclic peptide after deprotection. Ring contraction
for the synthesis of cyclic peptides by intramolecular
thiazolidine formation from linear unprotected peptide
precursors (Scheme 4) has recently been reported (Botti et
30 al, 1996). This procedure has the disadvantage of
incorporation of the thiazolidine ring, and an additional
stereo centre, into every sequence, and is not a generic
procedure suitable for a combinatorial library approach.



Scheme 4

Ring contraction chemistry for synthesis of cyclic peptides

5
Several other research groups have also utilised ring contraction approaches for the synthesis of cyclic peptides (Camamero and Muir, 1997; Shao et al, 1998). These procedures either require the presence of a Cys or
10 are restricted to cyclisation of peptides containing Gly at one of the termini, and are therefore not suitable for library development.

There is therefore a great need in the art for a mild, efficient, versatile synthetic strategy for the
15 synthesis of cyclic peptides. We have now found that by introducing substituents or other moieties which preorganise peptides for cyclisation, cyclic peptides can be efficiently synthesized under mild conditions both in solution and on resin. These moieties, which we have
20 termed *peptide cyclisation auxiliaries*, result in increased yields and purity of cyclic peptides. We have examined two approaches:

1. Positioning reversible *N*-amide substituents in the sequence.
- 25 2. Applying native ligation chemistry in an intramolecular sense.

We have evaluated these for their improvements in the solution and solid phase synthesis of small cyclic peptides.

30 We have systematically investigated the effects of preorganising peptides prior to cyclisation, and have developed new linkers to aid cyclic peptide synthesis. We have found surprising improvements in both yields and

purity of products compared to the prior art methods. The combination of these technologies provides a powerful generic approach for the solution and solid phase synthesis of small cyclic peptides.

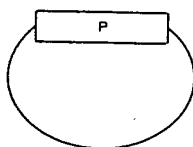
5 We have also developed linkers, and peptide cyclisation auxiliaries to aid cyclic peptide synthesis.

The ring contraction and *N*-amide substitution technology of the invention used in conjunction with the activated, safety catch, and backbone linker strategies of
10 the invention provide improved methods for the solid-phase synthesis of cyclic peptides.

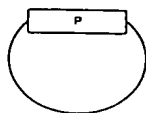
SUMMARY OF THE INVENTION

15 A feature of this invention is the combination of inducing flexibility in the peptide backbone, through reversible or irreversible *N*-substitution or forcing *cis* amide bond conformations via *cis*-amide bond surrogates, with novel ring contraction chemistry to preorganise peptides and facilitate the cyclisation reaction in
20 solution. Another feature of the invention is the option of combining one or more of these preorganising technologies with novel linkers which provide attachment between peptide and resin, to provide a solid phase strategy for the mild, efficient synthesis of cyclic
25 peptides or cyclic peptide libraries.

In its most preferred general aspect, this invention provides solution and solid-phase methods for the preparation of a cyclic peptide of the structure:

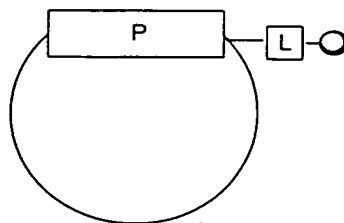


30
General Formula I



where is a cyclic peptide or peptidomimetic, in which the representation of the structure follows standard conventions with the C-terminus on the right hand side of P. It comprises between 1 to 15 monomers, preferably 1 to 10 monomers, more preferably 1 to 5 monomers. This may be a monocycle, bicycle or higher order cycle, and may comprise protected or unprotected monomers.

Another general aspect of the invention provides solid-phase methods for the synthesis of cyclic peptides or peptidomimetics of the structure:



15

General Formula II

where L is a linker unit, linking the cyclic peptide to the solid support . The linker L may be attached to any atom of the peptide, but is preferably attached to a backbone nitrogen or atom in the side chain of the monomer.

Thus, in a first aspect the invention provides a method of synthesis of cyclic peptides or cyclic peptidomimetic compounds, comprising the steps of:

a) inducing flexibility in the peptide or peptidomimetic compound by reversible N-substitution or by forcing a cis amide bond conformation using a cis-amide bond surrogate to facilitate cyclisation, and

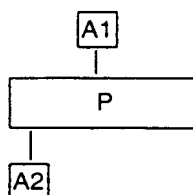
b) subjecting the cyclic peptide or peptidomimetic compound to a ring contraction reaction.

This ring contraction reaction may occur spontaneously, so that a separate reaction may not be required.

The method is applicable to both solution phase and solid phase synthesis.

In a preferred embodiment, this aspect of the invention provides a method for solution phase synthesis of a cyclic peptide of General Formula I, comprising the steps of:

-
- a) Preparing a linear peptide of General Formula III



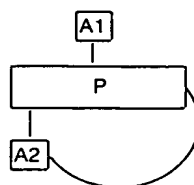
General Formula III

where P is a linear peptide of 10 to 15 monomers, preferably 1 to 10 monomers, most preferably 1 to 5 monomers.

A1 is one or more N-substituents, either reversible or non-reversible, on the peptide backbone, or is a chemical moiety that forces a *cis* conformation of the backbone, and

A2 is a covalently-bonded group of atoms comprising a reactive functionality to form an initial large cyclic peptide prior to ring contraction to the desired substituted cyclic peptide;

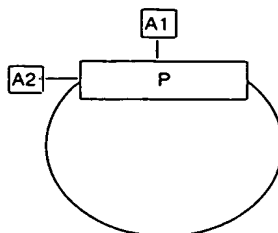
- b) Activating the C-terminus to form a cyclic peptide of General Formula IV:



General Formula IV

- 5 c) Permitting the peptide of General Formula IV to rearrange via a ring contraction reaction (which may occur spontaneously) to form a cyclic peptide of General Formula V; and optionally

10

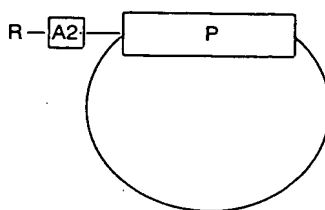


General Formula V

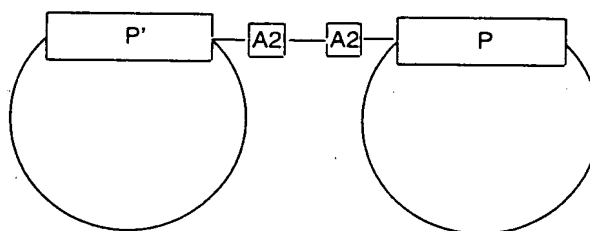
- 15 d) Subjecting the cyclic peptide of General Formula V to a deprotection reaction to remove the groups A1 and A2 to yield the desired cyclic peptide of General Formula I.

20 Optionally one or more of the groups A1 or A2 may be left attached to the peptide to provide a suitable point for attaching to a solid support, for derivatising with additional chemical functionality to improve library diversity, or for dimerisation or oligomerisation with other cyclic peptides or molecules, as illustrated below.

25



R = solid support or other chemical moiety



5

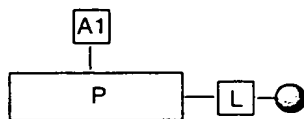
Alternatively ring contraction may lead to spontaneous elimination of A2.

10 Preferably A1 is a reversible N-substituent, such as 2-hydroxy-4-methoxybenzyl, 2-hydroxybenzyl or 2-hydroxy-6-nitrobenzyl substituents.

15 Preferably A2 comprises a nucleophile (eg. thiol or hydroxyl) that reacts rapidly with a C-terminus to form an initial large ring, which then contracts either spontaneously, or upon heating or additional chemical treatment (eg. addition of metal ions). A2 may be an irreversible substituent, may be removed after ring contraction, or may eliminate spontaneously, upon ring contraction. A2 also provides access to an additional site
20 for substitution to increase library diversity. A2 may also be any of the compounds of General Formula I described in our co-pending Australian provisional patent application filed on the same day as this application, entitled "Auxiliaries for Amide Bond Formation".

25 In a second aspect, the invention provides a method of solid phase synthesis of cyclic peptides, comprising the steps of:

a) synthesis of a linear peptide of General Formula VI, bound to a resin via a linker L,



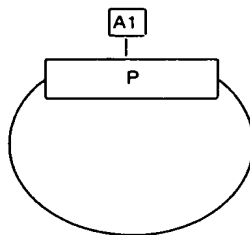
General Formula VI

5

where A1 and P are as defined above and L is a linker between any atom of the peptide and the resin, and

(b) either

- (i) subjecting the peptide (comprising either
10 protected or unprotected monomers) to cyclisation and concomitant cleavage from the resin to yield a cyclic peptide of General Formula VII,

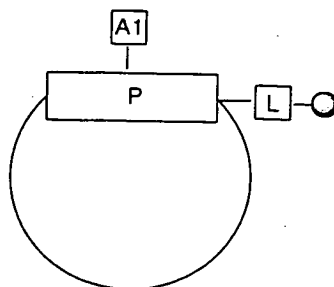


15

General Formula VII

followed by selective removal or derivatisation of A1 as described above, if necessary followed by side
20 chain deprotection of the peptide and removal of A1 to yield the desired cyclic peptide of General Formula I; or

- (ii) cyclisation of the peptide to yield a second resin-bound cyclic peptide of General Formula VIII,



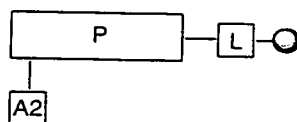
General Formula VIII

5 The person skilled in the art will appreciate that side chain deprotection of the peptide, removal of A1 and cleavage from the resin may be performed separately or concurrently. Removal of peptide protecting groups, A1 and
10 peptide of General Formula I.

Alternatively both a linker unit and A2 as described above are used.

Thus in another preferred embodiment, the invention provides a method of solid-phase synthesis of a
15 cyclic peptide, comprising the steps of:

a) preparing a linear resin-bound peptide of General Formula IX:

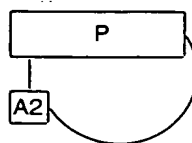


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General Formula IX

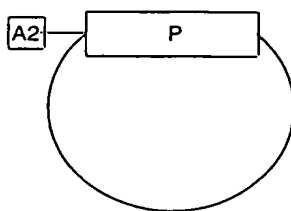
in which A2, P and L are as defined above;

b) subjecting the peptide IX to cyclisation
25 and concomitant cleavage from the resin to yield a cyclic peptide of General Formula X;



General Formula X

5 c) allowing the cyclic peptide X to undergo
ring contraction (which may occur spontaneously) to yield a
second cyclic peptide of General Formula XI, and



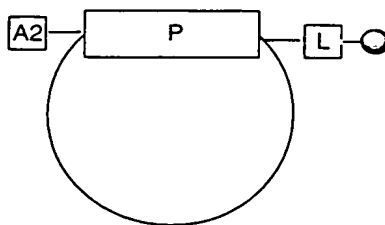
10

General Formula XI

 d) either derivatising the group A2, or removing
A2 to yield the desired cyclic peptide of General
15 Formula I.

 In another alternative the linear resin-bound
peptide of General Formula IX may be subjected to initial
cyclisation and ring contraction on the resin to yield a
resin-bound cyclic peptide of General Formula XII,

20



General Formula XII

25 and either

 (i) cleaved from the resin to yield an A2-
substituted cyclic peptide, or

(ii) deprotected and cleaved from the resin to yield a cyclic peptide of General Formula I.

Alternatively, the group A2 may be derivatised either in solid phase or in solution.

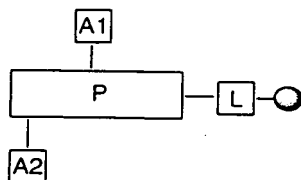
5 Again it will be appreciated that peptide deprotection, removal of A2 and cleavage from the resin may be performed separately or concurrently.

Most preferably the method of the invention utilises all three of

- 10 (i) N-substituents,
(ii) a covalently-bonded group of atoms which forms an initial large ring which subsequently contracts, and
(iii) synthesis on a solid support.

15 Therefore in a third aspect, the invention provides a method of solid phase synthesis of a cyclic peptide, comprising the steps of

- a) synthesis of a linear resin-bound peptide of General Formula XIII,

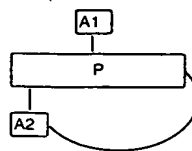


General Formula XIII

25 where A1, A2, P and L are as defined above;

- b) subjecting the peptide of General Formula XIII to cyclisation and concomitant cleavage from the resin to yield a cyclic peptide of General Formula XIV,

30



General Formula XIV

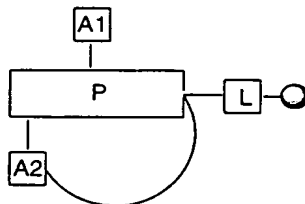
5 ~~e) subjecting the cyclic peptide of General~~
Formula XIV to ring contraction (which may be spontaneous),
and

d) cleaving the groups A1 and A2 to yield the
desired cyclic peptide of General Formula I.

10 Alternatively this aspect of the invention
provides a method of solid phase synthesis of cyclic
peptides, comprising the steps of;

a) synthesis of a linear resin-bound peptide
of General Formula XIII,

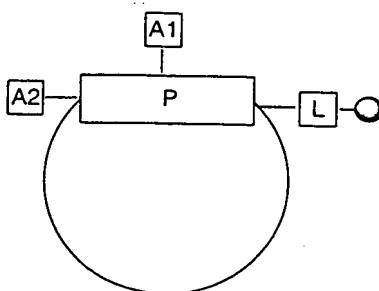
15 b) subjecting the linear peptide to
cyclisation on the resin to yield a cyclic peptide of
General Formula XV,



20

General Formula XV

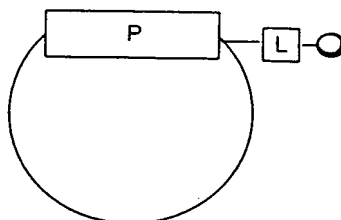
c) subjecting the cyclic peptide to ring
contraction (which may occur spontaneously) to yield a
25 cyclic peptide of General Formula XVI,



General Formula XVI

5 and either

d) cleaving groups A1 and A2 while the peptide is bound to the resin to yield a resin-bound cyclic peptide of General Formula II, or



General Formula II

10 e) subjecting the cyclic peptide to deprotection and concomitant cleavage from the resin to yield the desired cyclic peptide of General Formula I.

Once again it will be appreciated that peptide deprotection, removal of A2 and cleavage from the resin may be performed separately or concurrently.

20 For the purposes of this specification, the term "monomer" includes compounds which have an amino and carboxy terminus separated in a 1,2, 1,3, 1,4 or larger substitution pattern. This includes the 20 naturally-occurring α -amino acids in either the L or D configuration, 25 the biosynthetically-available amino acids not usually found in proteins, such as 4-hydroxy-proline, 5-hydroxylysine, citrulline and ornithine; synthetically-

derived α -amino acids, such as α -methylalanine, norleucine, norvaline, C α - and N-alkylated amino acids, homocysteine, and homoserine; and many others as known to the art. It also includes compounds that have an amine and

5 carboxyl functional group separated in a 1,3 or larger substitution pattern, such as β -alanine, γ -amino butyric acid, Freidinger lactam (Freidinger et al, 1982), the

bicyclic dipeptide (BTD) (Freidinger et al, 1982; Nagai and Sato, 1985), amino-methyl benzoic acid (Smythe and von

10 Itzstein, 1994), and others well known to the art.

Statine-like isosteres, hydroxyethylene isosteres, reduced amide bond isosteres, thioamide isosteres, urea isosteres, carbamate isosteres, thioether isosteres, vinyl isosteres and other amide bond isosteres known to the art are also

15 useful for the purposes of the invention. Thus the word "peptide" as used herein encompasses peptidomimetic compounds. Optionally the peptide may be protected with one or more protecting groups of the type used in the art (see for example Bodanszky, M., (1984), "Principles of

20 *Peptide Synthesis*", Springer-Verlag, Heidelberg).

A peptide is comprised of between one and fifteen monomers, preferably between one and ten monomers, more preferably one to five monomers.

The solid support may be of any type used for

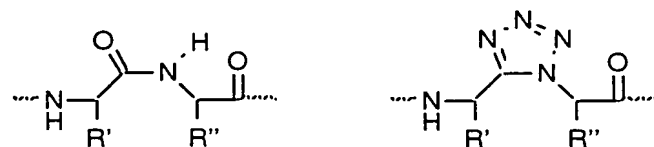
25 solid phase synthesis of peptides, peptidomimetics, oligonucleotides, oligosacharides or organic molecules. The solid support may be in the form of a bead, a pin or another such surface which is suitable for use in solid phase synthesis. A wide variety of suitable support

30 materials are known in the art. See for example Meldal, M., *Methods in Enzymology*, 1997 289 83-104. Commercially-available polystyrene supports, including aminomethyl-polystyrene, benzhydrylaminepolystyrene, polyethyleneglycol-polystyrene are especially suitable.

35 A "linker" means any covalently-bonded group of atoms which connects an atom or molecular fragment to another via covalent bonds. See for example Songster,

M.F., Barany. G., Methods in Enzymology, 1997 289 126-175. Typically the linker will comprise an optionally substituted allyl, aryl, alkylene group containing functionality, such as an ether, ester, amide, sulfonamide, sulfide, or sulfoxide functionality, within the linker.
5 Such a functionality will normally be used to create the connection between the two groups, or to separate the groups.

10 A "cis amide bond surrogate" is a chemical group, such as a tetrazole (Marshall et al, 1981), which forces a cis conformation.



15 For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

Coupling methods to form peptide bonds are well
20 known to the art. See for example Albericio and Carpino, 1997. When synthesising cyclic peptides in solution or upon a side chain or backbone attachment, the choice of activation can affect the yields and purity of cyclic material. For slow cyclisations the increased lifetime of
25 the intermediate activated linear peptide provides an opportunity for increased epimerisation at the C-terminal residue. The extent of epimerisation may be diminished by application of the azide method (Izumiya et al, 1981) or its modification using DPPA (Brady et al, 1983). However,
30 these methods are extremely slow, usually requiring many hours or even several days (Izumiya et al, 1981; Schmidt and Neubert, 1991; Heavner et al, 1991). In comparison with DPPA, TBTU (Knorr et al, 1989) and BOP (Castro et al, 1975) provide fast cyclisation, but may lead to C-terminal

epimerisation. The HOAt coupling reagents have recently been reported significantly to improve head-to-tail cyclisation of penta- and hexa-peptides with reduced epimerisation rates (Ehrlich et al, 1996).

5

Brief Description of the Figures

Figure 1 shows HPLC elution profiles of the crude product of solid phase synthesis of cyclo-D-G-(Cat)-R-G following cyclisation and concomitant cleavage from the resin (Profile A) and HPLC-purified cyclo-D-G-(Cat)-R-G synthesised in solution phase (Profile B).

Figure 2 shows an LC-MS profile of the crude filtrate obtained after HF cleavage and base cyclisation of a cyclic peptide synthesised using a safety catch linker of n=2.

Abbreviations used herein are as follows:

	DIEA	Diisopropylethylamine
20	DMF	dimethylformamide
	DMSO	dimethylsulphoxide
	DPPA	diphenylphosphoryl azide
	BOP	benzotriazo-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate
25	HOAt	7-aza-1-hydroxybenzotriazole
	HBTU	O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate
	HMB	2-hydroxy-4-methoxybenzyl
	HPLC	high performance liquid chromatography
30	ISMS	ion spray mass spectrometry
	LC-MS	liquid chromatography-mass spectrometry
	NMR	Nuclear Magnetic Resonance
	ROESY	rotating frame overhauser enhancement spectroscopy
35	r.t.	room temperature
	TOCSY	total correlated spectroscopy.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the figures.

5

Experimental

General Methods

Melting Points were determined on a Gallenkamp m.p. apparatus and are uncorrected. Solvent evaporation were carried out using a Büchi rotary evaporator. Deionised water was used throughout, and was prepared by a Milli-Q water purification system (Millipore-Waters). Screw-cap glass peptide synthesis reaction vessels (20 mL) with sintered glass filter frit were obtained from Embell Scientific Glassware (Queensland, Australia). An all-Kel-F apparatus (Peptide Institute) was used for HF cleavage. Argon, helium and nitrogen (all ultrapure grade) were from BOC gases (Queensland, Australia).

¹H NMR spectra were recorded on a Varian Gemini 300 spectrometer at 300 MHz, and chemical shifts are reported in δ parts per million down field from tetramethylsilane. Coupling constants (J) refer to vicinal proton-proton coupling. ¹³C NMR spectra were also recorded on a Varian Gemini spectrometer at 75.5 MHz. TOCSY and ROESY spectra were performed on a Büchi ARX 500 spectrometer.

Mass spectra were acquired on a PE-Sciex API-III triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 mL) were injected into a moving solvent (30 mL/min; 50/50 CH₃CN/0.05 % TFA) coupled directly to the ionisation source via a fused silica capillary interface (50 mm i.d. x 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyser through an interface plate and subsequently through an orifice (100-120 mm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range of 200 to

1000 daltons with a scan step size of 0.1 Da. Molecular masses were derived from the observed m/z values using the MacSpec 3.3 and Biomultiview 1.2 software packages (PE-Sciex Toronto, Canada).

5 Thin layer chromatography (Tlc) was performed on silica gel 60 F₂₅₄ plates (Merck Art 5735). The chromatograms were viewed under u.v. light and/or developed with iodine vapour. Preparative column chromatography was effected under pressure, using for normal phase Merck
10 Kieselgel 60 (Merck Art 7734). Analytical reverse phase HPLC were run using a C-18 Vydac column (218TP52022), while Semi-Preparative reverse phase HPLC was carried out using a C-18 Vydac column (218TP52022). Both columns were attached to a Waters HPLC apparatus fitted with a Holochrome U.V.
15 detector. Measurements were carried out at either $\lambda=214$ nm or 254 nm. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1 % aqueous TFA; B = 90 % CH₃CN, 10 % H₂O, 0.09 % TFA) at a flow rate of 0.25 mL/min (microbore), 1 mL/min (analytical) and 8 mL/min
20 (preparative).

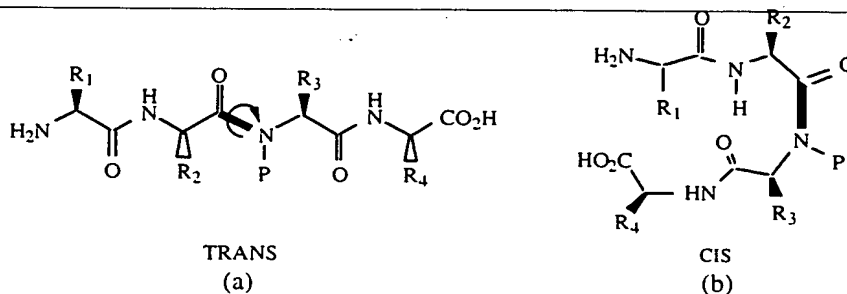
Materials

Boc-L-amino acids, Fmoc-L-aminoacids, Boc-Val-Polyaminomethylstyrene Resin, Merrifield resin, Boc-Gly-PAM
25 Resin, synthesis grade dimethylformamide (DMF), trifluoroacetic acid (TFA) and diisopropylethylamine (DIEA) were purchased from Auspep (Parkville, Australia) or Novabiochem (Alexandria, Australia). Chlorotriethyl Resin was purchased from Pepchem (Tubingen, Germany). HBTU and
30 BOP were purchased from Richelieu Biotechnologies (Montreal, Canada). Tris(2-carboxyethyl)phosphine hydrochloride salt (TCEP) was purchased from Strem Chemicals Inc. Newburyport MA. AR grade EtOAc, MeOH, CH₂Cl₂, CHCl₃, hexane, acetone and HPLC grade CH₃CN were
35 all obtained from Laboratory Supply (Australia), HF was purchased from CIG (Australia). All other reagents were AR grade or better, and were obtained from Aldrich or Fluka.

Example 1 Peptide Cyclisation Auxiliaries

Backbone substitution

- 5 *N*-substitution has the potential to alter the *cis-trans* equilibrium favouring more *cis* conformations and enhancing cyclisation yields:



- 10 We have examined the effect of the number and position of *N*-methylations on cyclisation yield of tetraglycine. Eight linear tetrapeptides were synthesised, including all permutations of glycine and sarcosine (*N*-methyl glycine) at the three *C*-terminal residues. These
15 are summarised in Table 3.

Table 3
Linear N-substituted Tetraglycines and
Corresponding Yields of Cyclisation

5	<u>Linear tetrapeptide</u>	<u>Yield of cyclisation</u>
	Gly-Gly-Gly-Gly	<1%
	Gly-Gly-Gly-Sar	8%
	Gly-Gly-Sar-Gly	11%
10	Gly-Sar-Gly-Gly	1%
	Gly-Gly-Sar-Sar	18%
	Gly-Sar-Gly-Sar	2%
	Gly-Sar-Sar-Gly	13% (16%*)
	Gly-Sar-Sar-Sar	~5%

15

* Yield of cyclisation for the corresponding N-HMB substituted linear tetraglycine, ie where sarcosine is replaced by [-N(HMB)-CH₂-CO-].

20

The yield for each cyclisation was calculated from the weight of isolated product. The results of this experiment suggest that N-substitution of the N-1 or N-2 position of a tetrapeptide significantly improves yields of cyclisation whereas N-substitution at the third residue has little effect. The effect of multiple substitution at two or more N-sites appears to be more or less additive. The best cyclisation result was obtained with the N-1 and N-2 substituted precursor Gly-Gly-Sar-Sar. However, from a synthetic point of view substitution at the N-1 position is less desirable, as this facilitates diketopiperazine formation at the dipeptide stage during assembly of the linear precursor. We have found that altering the position of the backbone substituent can significantly affect the ratio of monocycle over dimer or higher oligomers.

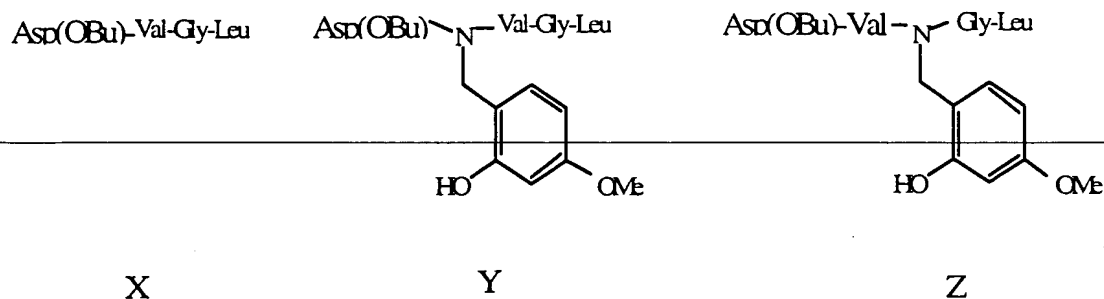
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30

35

We have extended this N-substitution approach to include reversible N-substitution. Three linear

precursors, the backbone unprotected peptide X and two backbone HMB-substituted analogues Y and Z, were prepared.



The three peptides were subjected to standard cyclisation protocols and the crude reaction mixtures analysed by HPLC and ISMS. The products (monomers and dimers) were further examined for epimerisation at the C-terminal leucine. Table 4 lists the products found and the corresponding yield of isolated material (% by weight).

Table 4
Yields of Isolated products from Cyclisation
of Tetrapeptides X, Y and Z

	X	Y	Z
Linear	—	10%	—
Monocycle (L-Leu)	—	8%	7%
Monocycle (D-Leu)	—	2%	16%
Dimer (L,D-Leu)	1%	—	8%
Dimer (L,L-Leu)	17%	19%	15%
Overall % D	3%	5%	43%

As expected, the unsubstituted tetrapeptide X generates dimers, with no detectable amounts of monocycle present as assessed by ISMS. Two dimers are found in a ratio of 1/10 as assessed by HPLC. The first eluting dimer

contains L-Leucine and D-Leucine in a ratio of 1/1. The second eluting dimer is formed from cyclisation of the all L-octapeptide. Considering that for cyclisation of peptide X, 0.5% D-Leu is observed and that a total yield of 18% was achieved, this equates to an overall epimerisation at the C-terminus of approximately 3% ($0.5/18 \times 100$).

On the other hand, both backbone-substituted tetrapeptides Y and Z generate a significant amount of cyclic tetrapeptide (monocycle), corroborating the N-Me study described above. As for peptide X, two dimers are formed [L-Leu/D-Leu and L-Leu/L-Leu] when cyclising peptide Y. For tetrapeptide Y a total of 80% of the separated monocycle contains L-Leu, but surprisingly for tetrapeptide Z a total of 70% of the separated monocycle contains D-Leu. For peptide Y about 5% D-Leucine is found in the total separated product, and for peptide Z 43% D-Leu is found. For tetrapeptide Z, this is equivalent to almost 100% racemisation (50% D-Leu : 50% L-Leu). In an attempt to minimise epimerisation of the C-terminus, cyclisation of tetrapeptide Z was performed with HATU instead of BOP. Under these conditions overall % D-leucine was halved.

Once epimerised, tetrapeptide Z cyclises more efficiently (16% D-Leu monocycle, no D-Leu/D-Leu dimer detected). Tetrapeptide Y is less reactive, as significant amounts of linear peptide are still present after three hours of activation. This may be explained by increased steric hindrance at the N-terminus.

We conclude that introduction of an HMB group on the middle amide nitrogen of the tetrapeptide X (ie. tetrapeptide Z) assists cyclisation, but significantly promotes epimerisation of the C-terminus. Substitution at the third amide nitrogen (tetrapeptide Y) assists cyclisation without increased epimerisation but reduces the reactivity of the peptide. In Example 3 below, we describe ring contraction chemistry that may help alleviate the epimerisation problems while enhancing cyclisation through N-substitution.

Experimental to Example 1

This section describes the experimental details for preorganising peptides prior to cyclisation via N-substitution.

Date in Table 3

Boc-Sar-Merrifield resin was prepared as follows:
Boc-Sar-OH (380 mg, 2 mmole) was dissolved in 2 mL H₂O containing Cs₂CO₃ (326 mg, 1 mmole). The mixture was lyophilised and residue taken up in DMF (5 mL). The solution is added to Merrifield resin (2.7 gr, 0.7 mmol/gr) and heated to 50°C overnight. The resin is filtered, washed and dried (3.05 gr, 0.65 mmole/gr). The tetrapeptides were assembled using *in situ* neutralisation protocols. After assembly the peptides were cleaved using HF/p-cresol (9/1) at 0°C for 1 hour. The HF was then evaporated and the product precipitated with cold ether (10 mL). After the ether washes (3 x 10 mL) the crude peptides were dissolved in water and purified by HPLC using 100% water (0.1%TFA) .

Cyclisation (Table 3)

The purified peptides (0.1 mmole) were dissolved in 100 mL DMF. BOP (133 mg, 0.3 mmole) was added followed by DIEA (0.5 mmole, 87 µL). After stirring overnight, the DMF was removed *in vacuo*, and the residues dissolved in acetonitrile/water (1/1) containing TFA (0.1%) and loaded on a reverse phase HPLC column. The isolated products from the HPLC run (10 minutes at 100% A, then 1% gradient to 50% B) were analysed by ISMS and analytical HPLC, dried and weighed. Yields were calculated from the weight of the isolated product.

Epimerisation Studies (Table 4)

The N-substituted linear peptides were synthesised on chloro-trityl resin. The HMB-protection

group was introduced via solid phase reductive alkylation of the *N*-terminus with 2-hydroxy-4-methoxybenzaldehyde (Ede et al, Tetrahedron Lett., 1996 37 9097). Acylation of the secondary amine was carried out by preactivating the
5 following Fmoc-protected residue using HOAT (2Eq.) and DIC (1Eq.) for 30 min in DMF and performing the reaction at 50°C for 12 hours. The peptide assembly was completed as described previously and linear peptide cleaved from the resin (1%TFA in DCM). All three peptides (all L-residues)
10 were purified by reverse phase HPLC prior to cyclisation.

Cyclisation

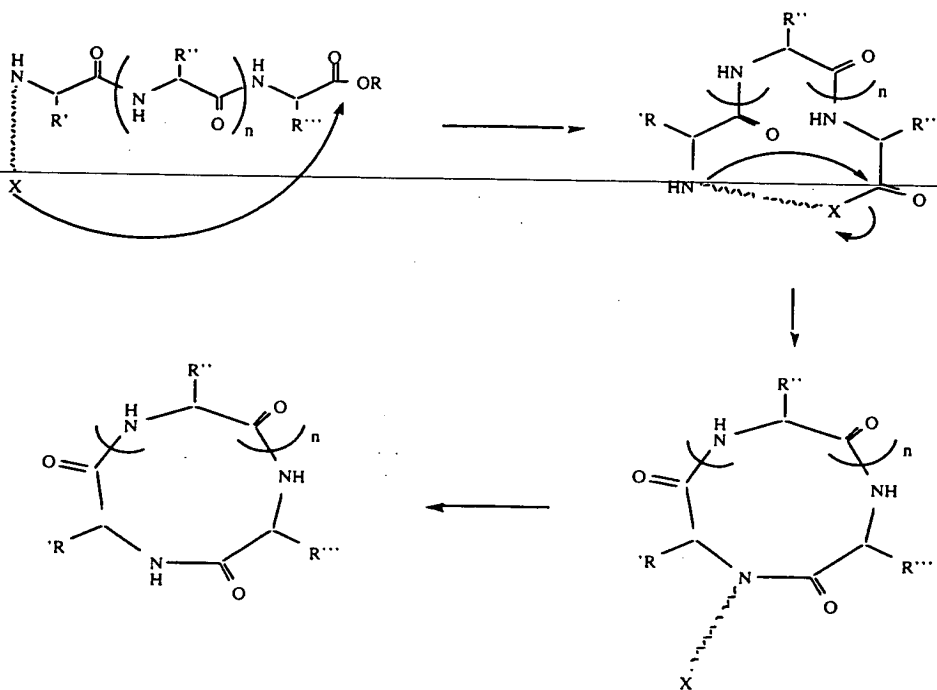
The purified peptides (0.1 mmole) were dissolved in DMF (100 mL). BOP (133 mg, 0.3 mmole) was added,
15 followed by DIEA (0.5 mmole, 87 µL). After 3 hours stirring the DMF was removed *in vacuo*, residues dissolved in acetonitrile/water (1/1) containing TFA (0.1%) and the solution loaded on a reverse phase HPLC column. The isolated products from the HPLC run (5 minutes at 80% A,
20 then 2% gradient to 100% B) were analysed by ISMS, analytical HPLC and epimerisation of leucine determined by amino acid analysis. Yields were calculated from the weight of the isolated product and the ratio of L/D from AA-analysis.

25

Example 2 Ring Contraction

Another approach to overcoming the problems in the solution and solid phase synthesis of small cyclic peptides is to utilise novel ring contraction chemistry.
30 As previously noted, the preferred extended conformation and rigidity of amide bonds is a problem in small peptide cyclisation. By initially forming a larger, more flexible ring, through the inclusion of a flexible "linker unit", the potential for end-to-tail cyclisation is enhanced by
35 increasing the effective concentration of the C- and *N*-terminus. The desired C- and *N*-termini are then appropriately positioned to "snap shut" in a ring

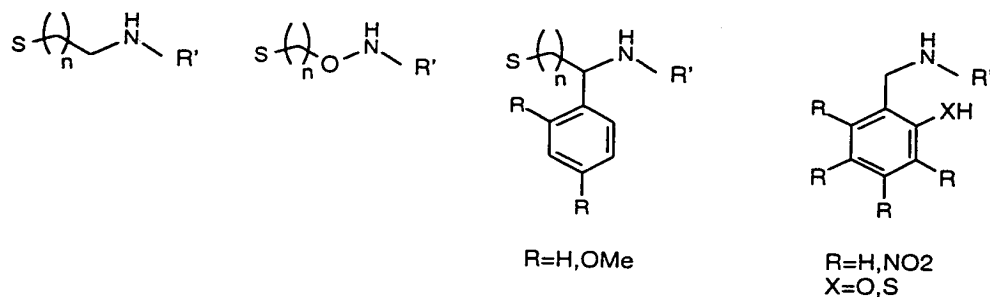
contraction reaction. This is shown schematically in Scheme 5.



5

Scheme 5
Ring contraction chemistry

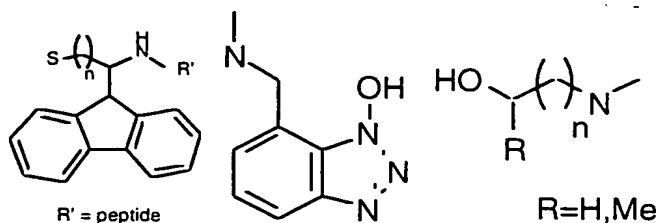
The ring contraction auxiliaries illustrated below are evaluated for this purpose.



Examples of ring contraction auxiliaries

15

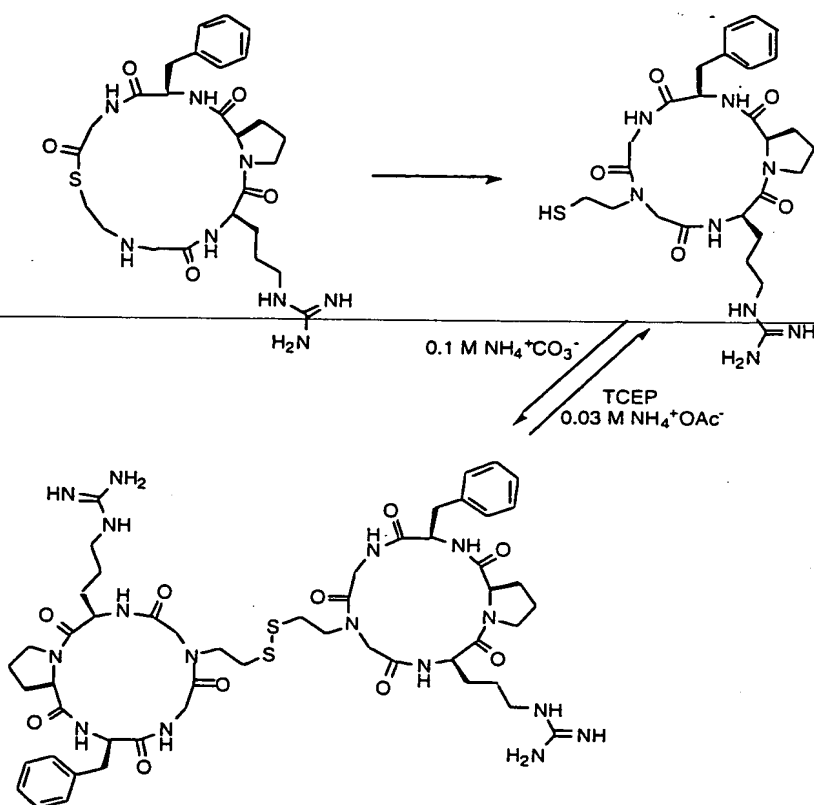
Additional auxiliaries include:



A subset of ring contraction auxiliaries

5 To examine the feasibility of the ring
contraction approach, we have synthesised a number of
linear pentapeptides carrying an ethane thiol group at the
N-terminus. The synthesis of the linear precursors was
achieved as illustrated in Scheme 6. Bromoacetic acid was
10 coupled to the N-terminus of the resin-bound tetrapeptide
using the symmetrical anhydride approach. The bromopeptide
was treated with a 2M solution of cystamine in DMSO and the
resulting peptide cleaved from the resin. The disulfide
moiety was further reduced using TCEP in an 0.1M ammonium
15 carbonate solution and the free sulfide purified by HPLC.
The sulfide was then subjected to standard cyclisation
conditions (ie 10^{-3} M in DMF, 3 eq. BOP, 5 eq DIEA).
Presumably, the initially formed thioester spontaneously
rearranges to the ethane thiol substituted cyclic peptide.
20 The resulting product was confirmed by NMR examination and
by the fact that the sulfide readily dimerises in DMF. The
dimer was isolated and characterised by ISMS and NMR.
Reduction of the dimer with TCEP reestablished the free
sulfide-peptide in quantitative yields.

25



Scheme 6

Synthesis and cyclisation of the linear ethane
thiol-substituted precursor for ring contraction

This process has several distinct advantages. The increased nucleophilicity of the thiol compared to the amine presumably results in rapid formation of the thioester, thereby significantly reducing the potential for epimerisation. The C- and N-termini are then appropriately positioned to snap shut in a ring contraction reaction.

In this example the ethane thiol group is irreversibly linked to the cyclic target. We have designed and tested other auxiliaries, outlined above, that allow cleavage of the auxiliary-peptide bond. The ring contraction in all the above-mentioned examples proceeds via a five or six-membered fused ring transition state.

Experimental to Example 2

This section describes the experimental details of the use of ring contraction concepts for the synthesis of small cyclic peptides.

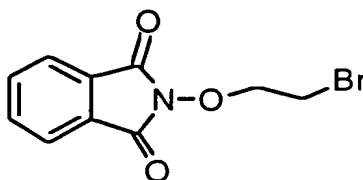
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Ring Contraction

Synthesis of Ring Contraction Auxiliaries

N-(2-Bromoethoxy)phthalimide

10



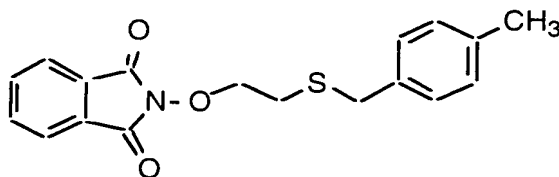
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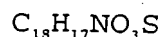
Mol. Wt.: 270.08

N-(2-Bromoethoxy)phthalimide was synthesised by a modification of the procedure of Bauer and Suresh (Bauer et al 1963). *N*-Hydroxyphthalimide (80 g, 0.49 mol), triethylamine (150 mL, 1.08 mol), and 1,2-dibromoethane (175 mL, 2.30 mol) were combined in DMF (575 mL) and stirred at room temperature overnight. Solids were filtered and washed with DMF and the filtrate was diluted with water (4.0 L) and the resulting precipitate filtered, dissolved in EtOAc (500 mL), and washed with 1 N HCl (2 x 100 mL), water (1 x 100 mL), and dried over MgSO₄. Volatiles were removed in vacuo, and the resulting solid recrystallised from 95% EtOH to give (9) as a white solid (87.1 g, 70%): mp. 94-96°C; lit. mp. 94-96°C. ¹H NMR (CDCl₃): δ 7.82 (m, 4H), 4.49 (t, 2H, J = 6.9 Hz), 3.65 (t, 2H, J = 6.9 Hz).

N-[2-[S-(4-Methylbenzyl)thio]ethoxy]phthalimide



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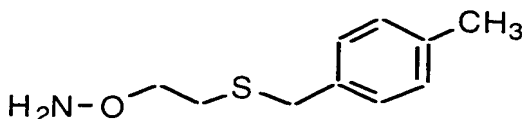


Exact Mass: 327.09

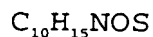
Mol. Wt.: 327.40

10 N-[2-[S-(4-Methylbenzyl)thio] ethoxy]phthalimide
was synthesised by a modification of the procedure of Canne
et al (Flanigan, 1971). Bromide (55.15 g 217 mmol),
4-methylbenzyl mercaptan (30 g, 217 mmol) and DIPEA
(38.55 mL, 217 mmol) were combined in acetonitrile (200 mL)
15 and stirred at room temperature for 72 h. Volatiles were
removed *in vacuo*, EtOAc (500 mL) added and filtered.
Solids were washed with EtOAc, and the organics were
combined and washed with 1 N HCl (2 x 200 mL), brine
(1 x 200 mL) and water (1 x 200 mL) and dried over MgSO₄.
20 Volatiles were removed *in vacuo* and the resulting solid
recrystallised from EtOAc : hexane, 1:1 to yield (10) as a
white solid (50.14 g, 71%): mp. 82-84°C; ¹H NMR (CDCl₃):
δ 7.80 (m, 4H), 7.18 (d, 2H, J = 8.0 Hz), 7.04 (d, 2H,
J = 8.0 Hz), 4.22 (t, 2H, J = 7.4 Hz), 3.75 (s, 2H),
25 2.79 (t, 2H, J = 7.4 Hz), 2.27 (s, 3H).

S-(4-Methylbenzyl)-2-(aminooxy)ethanediol



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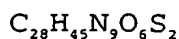
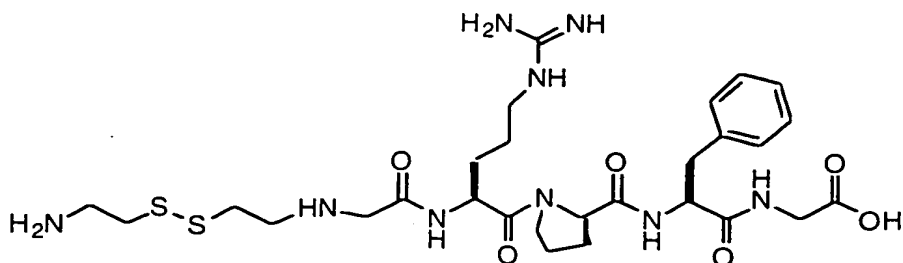


Exact Mass: 197.09

Mol. Wt.: 197.30

S-(4-Methylbenzyl)-2-(aminooxy) ethanediol was synthesised by a modification of the procedure by Osby et al (Cavelier-Frontin et al, 1993). The N-substituted pthalimide (20.0 g, 61.1 mmol) was suspended in a solution of 2-propanol (550 mL) and water (85 mL) and cooled to below 10°C. NaBH₄ (18.9 g, 252 mmol) was added portionwise so that the temperature did not exceed this temperature. The mixture was allowed to warm to room temperature and stirred overnight. Acetic acid (135 mL) was slowly added until the bubbling ceased, and the flask was stoppered and heated to 50°C for 3 h. Volatiles were removed *in vacuo*, and the resulting oil solution diluted with 1 N NaOH and extracted with EtOAc (4 x 200 mL). The hydroxylamine was then extracted into a solution of HCl (2N, 500 mL) and washed with EtOAc (2 x 250 mL). NaCO₃ was then added to the aqueous phase until bubbling ceased, and the hydroxylamine extracted into EtOAc (3 x 250 mL). The combined organic layers were washed with H₂O (2 x 250 mL) and dried over MgSO₄. Volatiles were removed *in vacuo*, and the resulting oil purified by flash chromatography (Hexane EtOAc, 3:1) to yield as a clear colourless oil (10.04g, 84%): ¹H NMR (CDCl₃): δ 7.21 (d, 2H, J = 8.0 Hz), 7.12 (d, 2H, J = 8.0 Hz), 5.40 (br s, 2H), 3.77 (t, 2H, J = 6.5 Hz), 2.71 (s, 2H), 2.64 (t, 2H, J = 6.5 Hz), 2.33 (s, 3H).

Application Of Ring Contraction Auxiliary (Scheme 6)
NH₂CH₂CH₂SSCH₂CH₂-Gly-Arg-Pro-Phe-Gly-OH

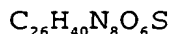
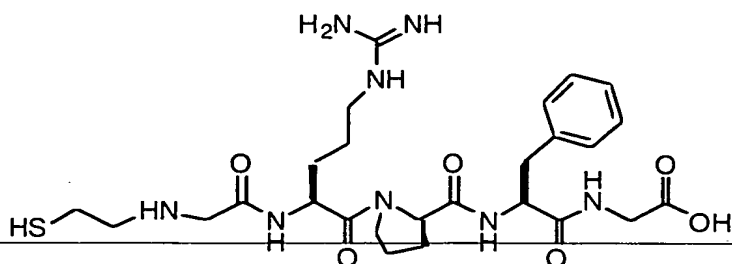


Exact Mass: 667.29

Mol. Wt. 667.85

The peptide $\text{NH}_2\text{CH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2$ -Gly-Arg-Pro-Phe-Gly-OH was synthesised in stepwise fashion from Boc-Gly-Pam resin (0.5 g, 0.5 mmol/g) by established methods, using in situ neutralisation/HBTU activation protocols for Boc chemistry. The Pmc protecting group was used for the Arg residue. Coupling reactions were monitored by quantitative ninhydrin assay and were typically >99.9%. After chain assembly was complete and the N^α -Boc group removed with neat TFA (2 x 1 min treatment) and neutralised with 10% DIEA in DMF (2 x 1 min treatment), the peptide was bromoacetylated by the method of Robey (Robey, F.A., Fields, R.L., Anal. Biochem., 1989 177 373-377). Bromoacetic acid (277.9 mg, 2.0 mmol) was dissolved in CH_2Cl_2 (2 mL), to which was added DIC (126.2 mg, 1 mmol). After activation for 10-15 min to form the symmetric anhydride, the mixture was diluted with DMF (2 mL), added to the peptide resin, and coupled for 30 min. The resin was washed with DMSO, and cystamine (2 M in DMF, 4 mL) was allowed to react with the bromoacetylated peptide resin for 16 h. The linear peptide was cleaved from resin by the addition of thiocresol: cresol, 1:1 (1 mL), followed by treatment with HF (10 mL) for 1 h at -5°C . After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous Et_2O and filtered to remove the scavengers. The peptide was dissolved in HOAc: H_2O , 1:19, filtered and the filtrate lyophilized. $\text{NH}_2\text{CH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2$ -Gly-Arg-Pro-Phe-Gly-OH was purified by semi-preparative HPLC (20-80% B over 60 min) to give the wanted material (79.6 mg 47%) yield. MS $[\text{M}+\text{H}]^+ = 668.1$ (expected 668.3).

HSCH₂CH₂-Gly-Arg-Phe-Gly-OH



Exact Mass: 592.28

Mol. Wt.: 592.71

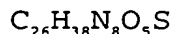
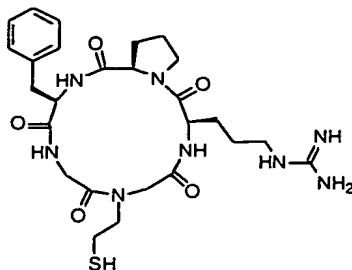
5

The disulfide (66.8 mg, 0.10 mmol) was dissolved in a 0.03 M solution of NH₄⁺OAc⁻ (20 mL).

10 Tris(2-carboxyethyl)phosphine hydrochloride salt (TCEP) (35.6 mg, 0.15 mmol) was added portionwise to the stirred solution at r.t. After a further 3h at this temperature the resulting mixture was lyophilized to give a white powder. The peptide HSCH₂CH₂-Gly-Arg-Phe-Gly-OH was
15 purified by semi-preparative HPLC (20-80% B over 60 min) to yield a white powder (40.1 mg, 68%); MS [M+H]⁺ = 593.1 (expected 593.3).

Cyclo-(SCH₂CH₂-Gly-Arg-Pro-Phe-Gly)

20



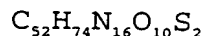
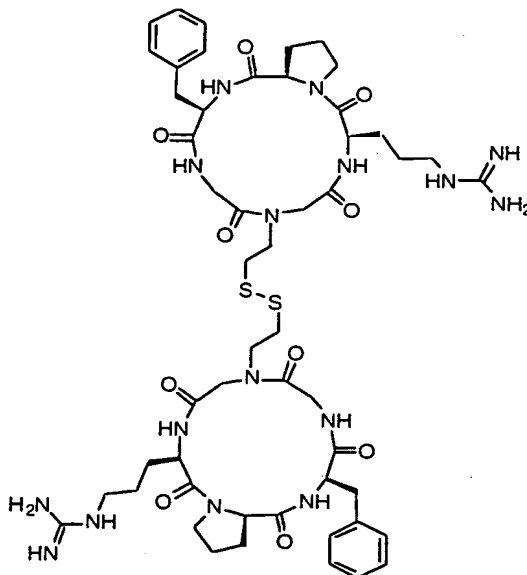
Exact Mass: 574.27

Mol. Wt.: 574.70

25

The linear peptide HSCH₂CH₂-Gly-Arg-Pro-Phe-Gly-OH (40.0 mg, 0.068 mmol) and BOP (88.4 mg, 0.2 mmol) was stirred in DMF (68 mL, 1x10⁻³ M) at -10°C. DIPEA (121 µL, 0.68 mmol) was added dropwise to the solution. The reaction was left to stir for a further 2 h at this temperature, before all volatiles were removed in vacuo. The peptide Cyclo-(SCH₂CH₂-Gly-Arg-Pro-Phe-Gly) was purified by semi-preparative HPLC (20-80% B over 60 min) to yield a white powder (12.2 mg, 31%); MS [M+H]⁺ = 743.2 (expected 743.4092).

Bis-[cyclo-Gly(CH₂CH₂S)-Arg-Pro-Phe-Gly]



Exact Mass: 1146.52

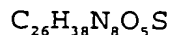
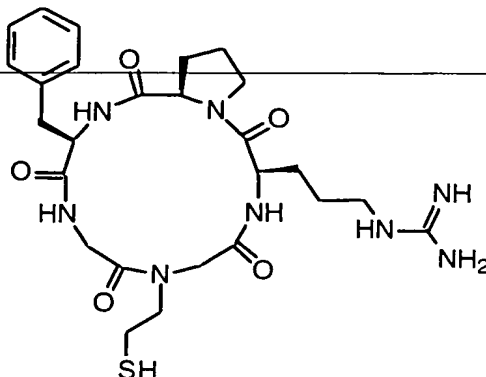
Mol. Wt.: 1147.38

The peptide Cyclo-(SCH₂CH₂-Gly-Arg-Pro-Phe-Gly) (12 mg, 0.016 mmol) was dissolved in a solution of Na₂HPO₄ (0.03 M) and stirred at room temperature overnight. The resulting solution was lyophilized to give a white powder. The peptide Bis-[cyclo-Gly(CH₂CH₂S)-Arg-Pro-Phe-Gly] () was purified by reverse phase HPLC (20-80% B over 60 min) to

yield a white powder (7.4 mg, 81%); MS $[M+2H]^{2+} = 574.22$ (expected 574.27).

Cyclo-(Gly(CH₂CH₂SH)-Arg-Pro-Phe-Gly)

5



Exact Mass: 574.27

Mol. Wt.: 574.70

10

The disulfide (7.4 mg, 6.50 μ mol) was dissolved in a 0.03 M solution of $NH_4^+Oac^-$ (20 mL). TCEP (4.75 mg, 20.0 μ mol) was added portionwise to the stirred solution at r.t. After a further 3h at this temperature the resulting mixture was lyophilized to give a white powder. The peptide Cyclo-(Gly(CH₂CH₂SH)-Arg-Pro-Phe-Gly) was purified by semi-preparative HPLC (20-80% B over 60 min) to yield a white powder (5.5 mg, 74%); MS $[M+H]^+ = 575.24$ (expected 575.28).

20

Example 3 Backbone Substitution and Ring Contraction in Solution.

The backbone substitution approach (Example 1) readily preorganises peptides prior to cyclisation and results in improved yields of target cyclic material. One drawback of this approach (Table 4) is increased racemisation of the C-terminus. The ring contraction approach (Example 2) results in the rapid formation of a larger cyclic peptide prior to ring contraction. The

combination of backbone substitution with ring contraction is expected to result in reduced racemisation, yet to achieve preorganising prior to cyclisation.

The array of compounds listed below is
 5 synthesized using this combined approach. These compounds all contain an N-substituent, in this case a methyl group, and a ring contraction auxiliary. The effects of this
 combination on the yield and purity of the product are evaluated, paying particular attention to the degree of C-
 10 terminal racemisation.

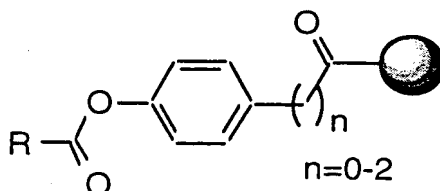


HX ~ = ring contraction
 auxiliary; X= O,S

A reversible N-substituent is also used, and the
 15 effects evaluated in the same way.

Example 4 Activated Linkers

Activated linkers of the general formula



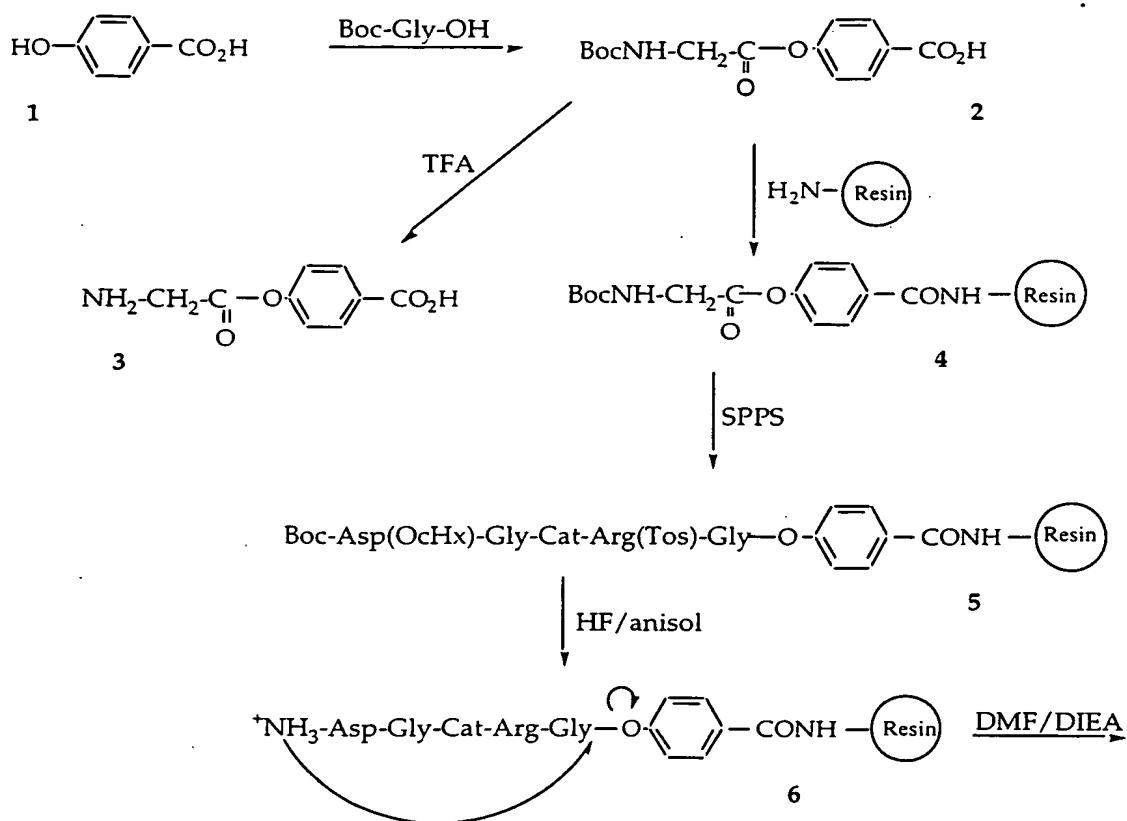
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have been evaluated for their stability during chain assembly and their lability in the final cyclisation

reaction. For the n=0 linker we have synthesised a series of constrained cyclic peptides, as illustrated in Table 5 below.

A general outline of the procedure used is shown in Scheme 7. The hydroxybenzoic acid (1) was acylated with Boc-Gly-OH. The resulting ester link was found to be stable to TFA treatment, as confirmed by treating compound (2) with TFA and subsequent 1H NMR analysis of the products (3).

10



Scheme 7

'Cyclisation by cleavage' experiments

15

Compound (2) was attached to amino-methylated resin (polystyrene) (substitution value (sv) = 0.21 mmol/g) using HBTU in DMF (Scheme 7). Peptide assembly was monitored by quantitative ninhydrin tests, and indicated successful assembly of the linear sequence. This was

20

confirmed by the increase in resin weight. The deprotection of the side chain protecting group was achieved by treatment with HF/anisole (9/1) at -5°C for 1 hour. After HF evaporation, the resin was washed with ether.

Cyclisation and accompanying cleavage was achieved by treatment with 10 equivalents DIEA in DMF for 3 days. The reaction mixture was worked up by filtration and the filtrate diluted with water and lyophilised. The crude lyophilised product was redissolved in acetonitrile/water (1/1) and further analysed by analytical and preparative HPLC.

The HPLC profile of the crude product is shown in Figure 1. The major component is the target peptide, as is evidenced by HPLC comparison and a coelution experiment with solution phase synthesised material. This result illustrates the potential power of this strategy in synthesising constrained cyclic peptides, particularly when considering the surprising purity of the crude material. The yields of cyclic material are given in Table 5.

Table 5

Yields of Cyclic Peptide Using Activated Linker

<u>Linear tetrapeptide</u>	<u>Yield of Cyclisation</u>
cyclo-[DG-Act-RG]	11%
cyclo-[DG-Amb-RG]	7%; 3% dimer
cyclo-[D-Amb-GRG]	5% monomer; 5% dimer

Example 4 Synthesis of Model Compounds Using Activated Linkers Cyclo-[DGActRG] (Table 5)

This section describes the experimental details for the synthesis of the activated linker and model peptides.

Linker Resin

The aminomethylated resin (2.38 gr, 0.5 mmole) was first washed with 10% DIEA in DMF (5 min) and then washed with DMF (3 x 5 ml). Hydroxybenzoic acid (276 mg, 2 mmole) was dissolved in 4 ml 0.5M HBTU in DMF and DIEA (400 µL, 2.3 mmol) added. The activated solution was then added to the neutralised resin. After 10 min the resin was drained and washed with DMF (3 x 5 mL). A solution of aqueous sodium hydroxide (1M, 2 mL) in DMF (4 mL) was added to the resin and mixed for 10 minutes. The sodium hydroxide treatment was repeated, and the resin washed with DMF/water (1/1) (3 x 5 mL) and then with DMF (3 x 5 mL).

Assembly of the Peptide

Boc-glycine was first coupled to the linker as follows. BocGlycine (350 mg, 2 mmole) was dissolved in 2 mL DCM and DIC (156 µL, 1 mmole) added. After 15 min the solution was diluted with 2 mL DMF, and added to the resin with DIEA (400 µL, 2.3 mmole). After 30 min, the resin was drained and washed with DMF (3 x 5 mL). The Boc group was then removed using neat TFA (2 x 1 min). The next residues were coupled using the following *in situ* neutralisation protocol: 2 mmole of the Boc-protected amino acid was dissolved in 4 mL of an 0.5M HBTU solution in DMF, and activated through addition of DIEA (460 µL, 2.6 mmole). The activated solution was then added to the resin and mixed for 10 minutes. The resin was drained and washed with DMF. Neat TFA (2 x 1 min) was used again for deprotection of the N-terminus. The following residues were coupled in series: Boc-Arg(Mts)OH, Boc-Gly-Cat-OH, Boc-Asp(OcHx)-OH.

Side-Chain Deprotection

After assembly the N-terminal Boc-group was removed with TFA as above, and the resin dried. The side chains were removed using HF treatment as follows: 1 gr of resin was mixed with 1 mL thioanisole and 9 mL of HF were

added. The mixture was stirred at -5°C for 1 hour and the HF removed under reduced pressure. The resin was washed with diethylether (3 x 20 mL) and dried.

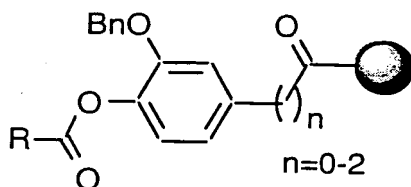
5 Cyclisation

The resin was stirred in DMF (10 mL) containing DIEA (100 μL) for 12 hours. The resin was filtered off and the DMF removed *in vacuo*. The residue was dissolved in a minimal amount acetonitrile/water (1/1) and loaded directly on a preparative reverse phase column for HPLC separation of the product. Cyclo-[DGCatRG] (27 mg, 11% yield from the starting resin) was obtained.

The same protocols were followed to assemble, deprotect and cyclise the following peptides:
cyclo-[DGAmBRG]: 7.6% yield (3% dimer); cyclo-[DAmBGRG] : 5% yield (5% dimer).

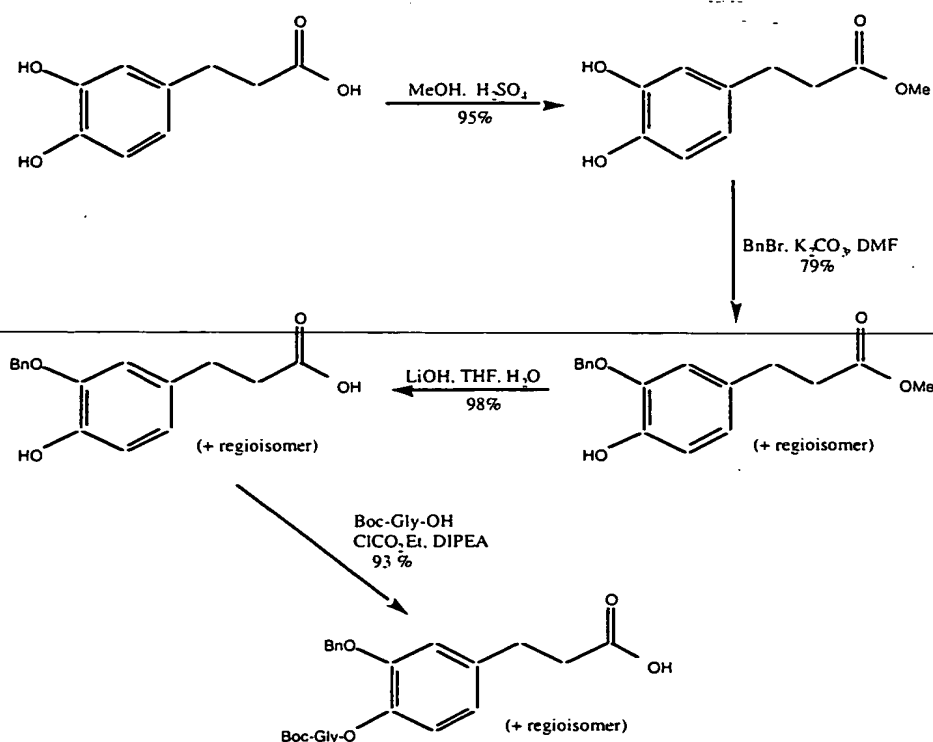
Example 5 Safety Catch Linkers

We have also evaluated the safety catch linkers of the general class



Examples of safety catch linkers

Activation of this linker is achieved by removal of the benzyl group. The safety-catch linker ($n=2$) was synthesised as shown in Scheme 8.



Scheme 8
Synthesis of safety catch linker

5

This safety-catch linker was attached to aminomethylated polystyrene using HBTU/DIPEA in DMF, then peptide assembly was accomplished using standard Boc protocols. Treatment of the resin with anhydrous HF in the presence of anisole as a scavenger at -5°C resulted in deprotection of the amino-acid side-chains, with concomitant removal of the benzyl group of the linker. The HF was evaporated and the resin was washed with diethyl ether to remove scavenger. Treatment of the resin with DIPEA in DMF for 48 h gave the crude cyclised product. An LC-MS profile of the crude cyclic material is shown in Figure 2. The major component is the desired cycle, and an appreciable amount of the cyclodimer is also present. Preparative-scale HPLC gave a mixture of the monomer and dimer, in an overall yield of approximately 50%.

10

15

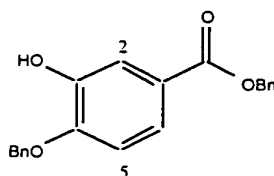
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Experimental to Example 5

This section describes the synthesis of the safety catch linker and model peptides.

5 Synthesis of Model Compounds using Safety Catch Linkers (Figures 6, n=0)

Benzyl 4-Benzyloxy-3-hydroxybenzoate



10

Benzyl bromide (1.50 cm³, 2.16 g, 12.6 mmol) was added to a stirred suspension of 3,4-dihydroxybenzoic acid (1.00 g, 6.49 mmol), potassium carbonate (1.97 g, 14.3 mmol) and a catalytic amount of tetrabutylammonium iodide in *N,N*-dimethylformamide (50 cm³). The suspension was stirred under nitrogen overnight then water (500 cm³) and 5% hydrochloric acid (50 cm³) were added, and the mixture was extracted with diethyl ether (3 x 100 cm³). The combined extracts were washed with water (3 x 100 cm³) and brine (100 cm³), then dried (Na₂SO₄) and evaporated to an orange oil. Flash column chromatography (eluent: 10-20% ethyl acetate in light petroleum) gave first benzyl 3,4-dibenzyloxybenzoate (168 mg, 6%), identical to that prepared above. Further elution then gave benzyl 4-benzyloxy-3-hydroxybenzoate (1.312 g, 60%) as a pale yellow oil. The position of the benzyloxy group was deduced from an n.O.e. observed between the proton at position 5 and the methylene protons of the benzyloxy group at position 4.

30

R_f 0.18 (20% EtOAc in light petroleum).

ν_{\max} (thin film, NaCl) 3600-3200, 1715, 1615, 1590 cm⁻¹.

¹H NMR (300 Hz, CDCl₃) 5.17, 2H, s, CH₂; 5.34, 2H, s, CH₂; 5.73, 1H, bs, OH; 6.95, 1H, d(*J* 8.2), H₅; 7.32-7.46, 10H, Ar-H; 7.65, 1H, dd(*J* 2.0, 10.6), H₆; 7.66, 1H, s, H₂; OH not observed.

5

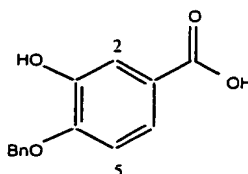
¹³C NMR (75 MHz, CDCl₃) 66.5, CH₂; 71.1, CH₂; 111.2, 115.9, 122.9, 123.6, 127.8, 128.0, 128.1, 128.2, 128.5, 128.6, 128.8, 135.5, 136.2, 145.4, 149.6, 166.0, CO₂.

10 Mass spectrum: 335 (MH⁺).

Found: *M* 334.1205; C₂₁H₁₉O₄ requires *M*⁺ 334.1205.

4-Benzyloxy-3-hydroxybenzoic Acid

15



A solution of lithium hydroxide hydrate (300 mg, 7.15 mmol) in water (15 cm³) was added dropwise to a stirred solution of benzyl 4-benzyloxy-3-hydroxybenzoate (1.177 g, 3.52 mmol) in tetrahydrofuran (35 cm³). The resulting emulsion was stirred overnight, by which time a clear, pale yellow solution had formed. More lithium hydroxide hydrate (300 mg, 7.15 mmol), water (25 cm³) and tetrahydrofuran (25 cm³) were added, and stirring was continued for 24 h. The tetrahydrofuran was removed under reduced pressure. Water (100 cm³) was added to the residual mixture, which was washed with diethyl ether (2 x 50 cm³), acidified to pH 1 with 5% HCl and extracted with dichloromethane (3 x 100 cm³). The combined extracts were washed with brine (50 cm³), dried (NaSO₄) and evaporated to give 4-benzyloxy-3-hydroxybenzoic acid as a white solid (638 mg, 74%). The diethyl ether washings were extracted with 1 M potassium hydroxide (2 x 25 cm³). The

combined extracts were acidified to pH 1 with 5% HCl and extracted with dichloromethane ($3 \times 100 \text{ cm}^3$). The combined extracts were dried over MgSO_4 and evaporated to give a further 119 mg of product (total yield 757 mg, 88%), m.p. 163-165°C.

ν_{max} (KBr disc) 3555, 3200-2400, 1676, 1619, 1592 cm^{-1} .

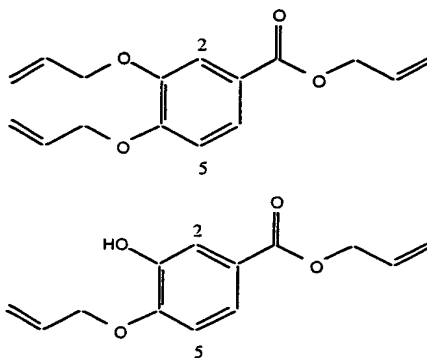
^1H NMR (300 Hz, CDCl_3) 5.19, 2H, s, CH_2 ; 5.71, 1H, br s, OH; 6.98, 1H, d(J 9.0), H5; 7.38-7.45, 5H, Ar-H; 7.67, 1H, dd(J 8.9, 2.1), H6; 7.68, 1H, d(J 2.0), H2; CO_2H not observed.

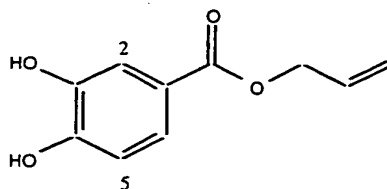
^{13}C NMR (75 MHz, CDCl_3) 71.2, CH_2 ; 111.2, 116.3, 122.6, 123.5, 127.9, 128.7, 128.9, 135.4, 145.5, 150.2, 170.6, CO_2 .

Mass spectrum: 245 (MH^+).

Found: M 244.0740; $\text{C}_{14}\text{H}_{12}\text{O}_4$ requires M^+ 244.0736.

Allylation of 3,4-Dihydroxybenzoic acid: Preparation of Propen-2-yl 3,4-Bis(propen-2-yloxy)benzoate, Propen-2-yl 3-hydroxy-4-(propen-2-yloxy)benzoate and Propen-2-yl 3,4-dihydroxybenzoate





Allyl bromide (1.18 cm³, 1.65 g, 13.6 mmol) was added to a stirred suspension of 3,4-dihydroxybenzoic acid (1.00 g, 6.49 mmol) and potassium carbonate (1.97 g, 14.3 mmol) in dry *N,N*-dimethylformamide (50 cm³). After stirring overnight under an atmosphere of nitrogen, the mixture was poured into water (500 cm³), acidified with 5% hydrochloric acid and extracted with ethyl acetate (3 x 100 cm³). The combined extracts were washed with water (3 x 100 cm³) and brine (50 cm³), then dried over MgSO₄ and evaporated to a brown oil which was purified by flash column chromatography (eluent: 10-50% ethyl acetate in light petroleum). The first compound to elute was propen-2-yl 3,4-bis(propen-2-yloxy)benzoate as a pale yellow oil (460 mg, 26%).

R_f 0.43 (20% EtOAc in light petroleum).

*v*_{max} (thin film, NaCl) 1718, 1648, 1600, 1270 cm⁻¹.

¹H NMR (300 Hz, CDCl₃) 4.64, 2H, dt(*J* 1.6, 5.2), OCH₂; 4.66, 2H, dt(*J* 1.7, 5.1), OCH₂; 4.79, 2H, dt(*J* 1.5, 5.7), OCH₂; 5.24-5.47, 6H, m, 3x =CH₂; 5.97-6.15, 3H, m, 3x =CH; 6.88, 1H, d(*J* 8.5), H5; 7.58, 1H, d(*J* 2.0), H2; 7.67, 1H, dd(*J* 2.0, 8.4), H6.

¹³C NMR (75 MHz, CDCl₃) 65.3, 69.6 and 69.8, 3x CH₂O; 112.3 and 114.6, C2 and C5; 117.9, 117.9 and 118.0, 3x =CH₂; 122.7, C1; 123.7, C6; 132.4, 132.6 and 132.9, 3x CH=CH₂; 147.9, C3; 152.5, C4; 165.9, C=O.

Mass spectrum: 275 (MH⁺), 217 (MH-C₃H₅O)

Found: M 274.1204; $C_{16}H_{18}O_4$ requires M^+ 274.1205.

Next to elute was *propen-2-yl 3-hydroxy-4-(propen-2-yloxy)benzoate* as a pale pink oil (782 mg, 51%).

R_f 0.26 (20% EtOAc in light petroleum).

ν_{\max} (thin film, NaCl) 3422 br, 1718, 1616, 1590, 1508 cm^{-1} .

10

^1H NMR (300 Hz, CDCl_3) 4.67, 2H, dt(J 5.5, 1.4), OCH_2 ; 4.79, 2H, dt(J 5.5, 1.5), OCH_2 ; 5.25-5.45, 4H, m, $2\times\text{CH}_2$; 5.70, 1H, s, OH; 5.96-6.12, 2H, m, $2\times\text{CH}=\text{CH}_2$; 6.87, 1H, d(J 8.7), H5; 7.62, 1H, dd(J 7.7, 2.2), H6; 7.63, 1H, br s, H2.

15

^{13}C NMR (75 MHz, CDCl_3) 65.4, OCH_2 ; 69.8, OCH_2 ; 111.1 and 115.8, C2 and C5; 118.0 and 119.0, $2\times\text{CH}_2$; 122.7, C6; 123.5, C1; 132.1 and 132.4, $2\times\text{CH}$; 145.4, C3; 149.4, C4; 165.9, C=O.

20

Mass spectrum: 235 (MH^+), 177 ($\text{MH}-\text{C}_3\text{H}_5\text{O}$), 149 ($\text{MH}-\text{C}_4\text{H}_5\text{O}_2$).

Found: M 234.0892; $C_{13}H_{14}O_4$ requires M^+ 234.0892.

25

Last to elute was *propen-2-yl 3,4-dihydroxybenzoate* as a pale yellow semi-solid (80.2 mg, 6%).

R_f 0.30 (50% EtOAc in light petroleum).

30

ν_{\max} (KBr disc) 3468br, 3344br, 1693, 1613, 1445, 1300 cm^{-1} .

^1H NMR (300 Hz, CDCl_3) 4.78, 2H, d(J 5.4), OCH_2 ; 5.27, 1H, br d(J 10.5), $=\text{CHH}$; 5.39, 1H, br d(J 18.6), $=\text{CHH}$; 5.94-6.07, 1H, m, $\text{CH}=\text{CH}_2$; 6.90, 1H, d(J 7.8), H5; 7.56, 1H, d(J 7.8), H6; 7.64, 1H, br s, H2; OHs not observed.

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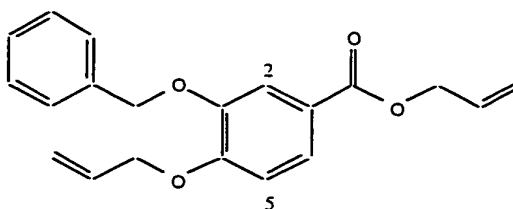
¹³C NMR (75 MHz, CDCl₃) 65.7, OCH₂; 114.8 and 116.3, C2 and C5; 118.3, =CH₂; 122.1, C1; 123.7, C6; 132.1, =CH; 143.3, C3; 149.2, C4; 166.9, C=O.

5 Mass spectrum: 195 (MH⁺).

Found: *M* 194.0578; C₁₀H₁₀O₄ requires *M*⁺ 194.0579.

Propen-2-yl 3-Benzyloxy-4-(propen-2-yloxy)benzoate

10



Benzyl bromide (0.440 cm³, 634 mg, 3.70 mmol) was added to a stirred mixture of propen-2-yl 3-hydroxy-4-(propen-2-yloxy)benzoate (782 mg, 3.34 mmol) and potassium carbonate (553 mg, 4.00 mmol) in *N,N*-dimethylformamide (30 cm³). The mixture was stirred under nitrogen overnight, then poured into water (300 cm³) and extracted with ethyl acetate (3 x 100 cm³). The combined extracts were washed with water (3 x 50 cm³) and brine (50 cm³), then dried over MgSO₄ and evaporated to a colourless oil. This was dissolved in dichloromethane and filtered through a plug of silica. Evaporation of the filtrate gave propen-2-yl 3-benzyloxy-4-(propen-2-yloxy)benzoate as a colourless oil (1.096 mg, 100%).

*R*_f 0.42 (20% EtOAc in light petroleum).

*v*_{max} (thin film, NaCl) 1714, 1600, 1514, 1428 cm⁻¹.

30

¹H NMR (300 Hz, CDCl₃) 4.67, 2H, dt(*J* 5.2, 1.6), =CH-CH₂; 4.79, 2H, dt(*J* 5.6, 1.5), =CH-CH₂; 5.19, 2H, s, PhCH₂; 5.29, 2H, ddt(*J* 10.2, 2.8, 1.5), =CH₂; 5.41, 2H, ddt(*J*

17.2, 3.1, 1.6), =CH₂; 5.96-6.15, 2H, m, 2x =CH; 6.91, 1H, d(*J* 8.5), H5; 7.30-7.49, 5H, PhCH₂; 7.66, 1H, d(*J* 2.0), H2; 7.69, 1H, dd(*J* 8.4, 2.0), H6.

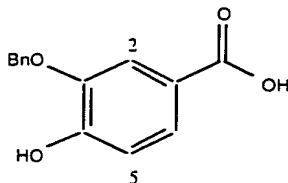
5 ¹³C NMR (75 MHz, CDCl₃) 66.3, 69.6 and 71.0, 3x CH₂O; 112.5 and 115.2, 2x =CH; 117.9, 2x =CH₂; 122.7, C1; 127.3, 127.9, 128.5, 132.4 and 132.6, 5x CH; 136.7; 148.0 and 152.7, C3 and C4; 165.9, C=O.

10 Mass spectrum: 325 (MH⁺).

Found: *M* 324.1361; C₂₀H₂₀O₄ requires *M*⁺ 324.1362.

3-Benzyloxy-4-Hydroxybenzoic Acid

15



A mixture of propen-2-yl 3-benzyloxy-4-(propen-2-yloxy)benzoate (1.0356 g, 3.19 mmol), tris(triphenylphosphine)rhodium chloride¹ (204 mg, 0.22 mmol) and 1,4-diazabicyclo[2.2.2]octane (74 mg, 0.66 mmol) in ethanol (18 cm³) and water (2 cm³) was heated under reflux under an atmosphere of nitrogen for 16 h. The cooled mixture was poured into 1 M hydrochloric acid (100 cm³), stirred for 60 min, then extracted with dichloromethane (3 x 100 cm³). The combined extracts were dried over MgSO₄ and evaporated to an orange solid which was purified by flash column chromatography (eluent: 1:1 EtOAc:light petroleum) to give 3-benzyloxy-4-hydroxybenzoic acid as an orange solid (650 mg, 83%), m.p. 167.2-171.3°C

R_f 0.25 (50% EtOAc in light petroleum).

ν_{\max} (KBr disc) 3528, 3200-2600, 1700, 1673, 1611 cm^{-1} .

^1H NMR (300 Hz, CDCl_3) 5.18, 2H, s, CH_2 ; 6.13, 1H, br s, OH; 7.00, 1H, d(J 8.3), H5; 7.37-7.50, 5H, m, Bn-H; 7.71, 1H, d(J 1.9), H2; 7.75, 1H, dd(J 1.9, 8.3), H6; CO_2H not observed.

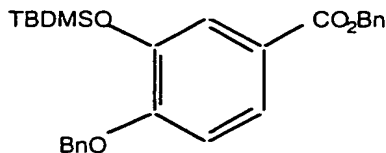
^{13}C NMR (75 MHz, CDCl_3) 71.4, CH_2 ; 113.5, 114.4, 121.2, 125.5, 128.1, 128.7, 128.8, 135.6, C1; 145.4 and 151.0, C3 and C4; 171.0, C=O

Mass spectrum: 245 (MH^+)

Found: M 244.0731; $\text{C}_{14}\text{H}_{12}\text{O}_4$ requires M^+ 244.0736.

1. Corey, E.J. and Suggs, J.W., J. Org. Chem., 1973 38 3224.

Benzyl 3-(*tert*-Butyldimethylsilyloxy)-4-benzyloxybenzoate



20

A solution of *tert*-butyldimethylsilyl chloride (579 mg, 3.84 mmol) in dichloromethane (10 cm^3) was added to a stirred solution of benzyl 4-benzyloxy-3-hydroxybenzoate (642.3 mg, 1.92 mmol) and imidazole (327 mg, 4.80 mmol) in dichloromethane (15 cm^3). A thick precipitate formed immediately. After 1 h the mixture was poured into water (50 cm^3). The layers were shaken and separated and then the aqueous phase was further extracted with dichloromethane (2 x 50 cm^3). The combined extracts were washed with brine (50 cm^3) then dried (Na_2SO_4) and evaporated to a pale yellow oil. This was taken up in 20% ethyl acetate in petroleum ether and filtered through a plug of silica. Evaporation of the filtrate gave the title

compound as a pale yellow oil (936 mg) which was used immediately for the next step.

R_f 0.49 (20% EtOAc in hexane).

5

ν_{\max} (NaCl film) 1718, 1599, 1509, 1427, 1290, 1213, 837 cm^{-1} .

1
10 ^1H NMR (300 Hz, CDCl_3) 0.11, 6H, s, SiMe_2 ; 0.96, 9H, s, CMe_3 ; 5.10, 2H, s, CH; 5.33, 2H, s, CH_2 ; 6.92, 1H, d(J 8.7), H5; 7.31-7.45, 10H, 10 x Bn-H; 7.59, 1H, d(J 1.5), H2; 7.67, 1H, dd(J 2.2, 8.9), H6.

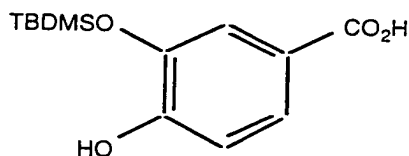
13
15 ^{13}C NMR (75 MHz, CDCl_3) -4.6, SiMe_2 ; 18.4, CMe_3 ; 25.6, CMe_3 ; 66.4 and 70.7, $2\times\text{CH}_2$; 112.5, 122.2, 124.3, 127.8, 127.9, 128.1, 128.2, 128.3, 128.5, 136.2, 136.4, 144.9, 154.5, Ar-C; 166.1, C=O.

Mass spectrum: (MH^+)

20

Found: M ; $\text{C}_{27}\text{H}_{32}\text{O}_4\text{Si}$ requires M^+

3-(*tert*-Butyldimethylsilyloxy)-4-hydroxybenzoic Acid



25

30 A solution of the crude silyl ether (936 mg, 2.09 mmol) in ethanol (50 cm^{-1}) containing 10% palladium-on-carbon (80 mg) was shaken under an atmosphere of hydrogen at 25 p.s.i. for 48 h. The mixture was filtered through celite and evaporated, then the residue was taken up in ethyl acetate and filtered through a plug of silica to give the title compound as a pale green oil (424 mg, 76%).

ν_{\max} (NaCl film) 3516, 3400-2600, 1682, 1597, 1298 cm^{-1} .

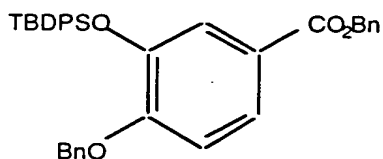
¹H NMR (300 Hz, CDCl_3) 0.33, 6H, s, SiMe_2 ; 1.04, 9H, s, CMe_3 ; 6.05, 1H, brs, OH; 6.99, 1H, d(J 8.4), H5; 7.60, 1H, d(J 2.1), H2; 7.73, 1H, dd(J 1.9, 8.5), H6.

¹³C NMR (75 MHz, CDCl_3) -4.4, SiMe_2 ; 18.2, CMe_3 ; 25.6, CMe_3 ; 114.6, 119.3, 121.3, 125.5, 142.1, 152.5, 6xArC; 171.9, C=O.

Mass spectrum: (MH^+)

Found: M ; $\text{C}_{13}\text{H}_{20}\text{O}_4\text{Si}$ requires M^+

Benzyl 4-benzyloxy-3-(tert-Butyldiphenylsilyloxy)benzoate



A solution of tert-butyldiphenylsilyl chloride (850 mg, 3.09 mmol) in dichloromethane ($10 \text{ cm}^3 + 5 \text{ cm}^3$ rinse) was added to a stirred solution of benzyl 4-benzyloxy-3-hydroxybenzoate (827 mg, 2.47 mmol) and imidazole (421 mg, 6.18 mmol) in dichloromethane (15 cm^3). After a few minutes a precipitate formed. The mixture was stirred overnight under an atmosphere of nitrogen, then was poured into water (50 cm^3). The layers were shaken and separated, then the aqueous phase was further extracted with dichloromethane ($2 \times 50 \text{ cm}^3$). The combined extracts were washed with brine (50 cm^3) and evaporated to a pale yellow oil. This was filtered through a short silica column and eluted with 20% ethyl acetate in petroleum ether. Evaporation of the filtrate gave benzyl 3-(tert-butyldiphenylsilyloxy)-4-benzyloxybenzoate (1.646 g) as a

very pale yellow oil, containing some *tert*-butyldiphenylsilanol, which was used directly for the next step.

R_f 0.37 (20% EtOAc in hexane).

5

ν_{\max} (NaCl, thin film) 1715, 1599, 1510, 1427, 1291 cm^{-1} .

1

^1H NMR (300 Hz, CDCl_3) 1.13, 9H, s, CMe_3 ; 4.93, 2H, s, CH_2O ; 5.25, 2H, s, CH_2O ; 6.84, 1H, d(J 8.9), H5; 7.21-7.43, 16H, m, 16xAr-H; 7.55, 1H, d(J 2.1), H2; 7.63, 1H, dd(J 2.1, 8.4), H6; 7.70-7.79, 4H, m, 4xAr-H.

10

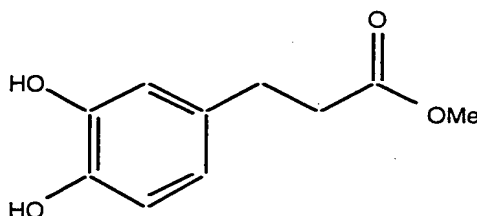
^{13}C NMR (75 MHz, CDCl_3) 19.7, CMe_3 ; 26.6, CMe_3 ; 66.2 and 70.3, 2x CH_2O ; 112.4, 121.4, 122.6, 124.1, 127.4, 127.5, 127.7, 127.8, 127.9, 128.3, 128.4, 129.7, 133.1, 134.8, 135.3, 136.2, 144.7 and 153.8, 18xAr-C; 165.9, C=O.

15

Mass spectrum: (MH^+).

20 Synthesis of Model Compounds Using Safety Catch Linker.

Methyl 3-(3,4-Dihydroxyphenyl)Propionate



25

A solution of 3-(3,4-dihydroxyphenyl)propionic acid (1.00 g, 5.49 mmol) and concentrated H_2SO_4 (10 drops) in methanol (25 cm^3) was heated under reflux overnight. The solvent was evaporated and the residue was shaken with water (50 cm^3) and extracted into CHCl_3 (3 x 50 cm^3). The combined extracts were dried (Na_2SO_4) and evaporated to gave the methyl ester a pale yellow oil which crystallised

30

on standing (1.12g, 100%), m.p. 71.9-74.1°C (lit.¹ m.p. 74-76°C).

n_{\max} (KBr disc) 3485, 3311, 1712 cm^{-1} .

5

¹H NMR (300 Hz, CDCl_3) 2.61, 2H, t(J 7.5), CH_2CO_2 ; 2.83, 2H, t(J 7.6), ArCH_2 ; 3.69, 3H, s, OMe; 5.40, 2H, br s, 2xOH; 6.61, 1H, dd(J 2.1, 8.1), H6; 6.71, 1H, d(J 2.0), H2; 6.77, 1H, d(J 8.1), H5.

10

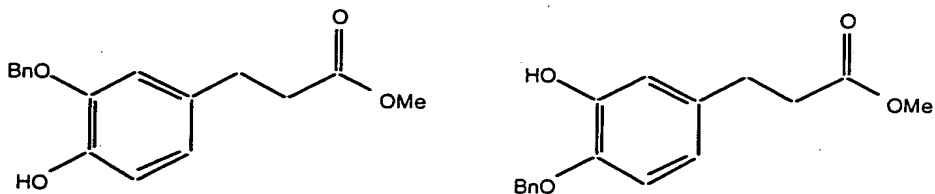
¹³C NMR (75 MHz, CDCl_3) 30.2 and 35.9, 2x CH_2 ; 51.9, OMe; 115.4, C2 and C6; 120.5, C5; 133.2, C1; 142.1 and 143.6, C3 and C4; 174.3, C=O.

15 Mass spectrum: (MH^+).

Found: M 196.0739; $\text{C}_{10}\text{H}_{12}\text{O}_4$ requires M^+ 196.0736.

1. Chem. Ber., 1953, 86, 190.

20 **Methyl 3-(3-Benzyloxy-4-hydroxyphenyl)Propionate and Methyl 3-(4-Benzyloxy-3-hydroxyphenyl)Propionate**



25 Benzyl bromide (0.606 cm^3 , 872 mg, 5.20 mmol) was added to a stirred suspension of methyl 3-(3,4-dihydroxyphenyl)propionate (1.000 g, 5.10 mmol), K_2CO_3 (845 mg, 6.12 mmol) and a catalytic amount of tetrabutylammonium iodide in DMF (25 cm^3). The suspension was stirred
30 overnight under an atmosphere of nitrogen. Water (500 cm^3) and 5% HCl (50 cm^3) were added, and the mixture was extracted with diethyl ether (3 x 100 cm^3). The combined extracts were washed with water (3 x 100 cm^3) and brine

(100 cm³), then dried (Na₂SO₄) and evaporated to a brown oil which was purified by flash chromatography (5-20% EtOAc in petrol) to give a 1:1 mixture of the monobenzyl ethers as a colourless oil (1.150 g, 79%)

5

n_{max} (NaCl thin film) 3446, 1732, 1592, 1514 cm⁻¹.

1

¹H NMR (300 Hz, CDCl₃) 2.60, 4H, t(*J* 7.4), 2xCH₂CO₂; 2.87, 2H, t(*J* 7.8), CH₂CH₂CO₂; 2.89, 2H, t(*J* 7.7), CH₂CH₂CO₂; 10 3.67, 3H, s, OMe; 3.68, 3H, s, OMe; 5.08, 2H, PhCH₂; 5.09, 2H, PhCH₂; 6.67, 1H, dd(*J* 8.2, 2.1), H₆; 6.73, 1H, dd(*J* 8.0, 1.6), H₆; 6.81, 2H, br s, H_{2,2}; 6.82, 1H, d(*J* 8.0), H₅; 6.88, 1H, d(*J* 8.2), H₅; 7.30-7.50, 10H, Ar-H.

15 ¹³C NMR (75 MHz, CDCl₃) 30.3, 30.6, 35.7 and 36.0, 2xCH₂CH₂; 51.5, 2xOMe; 71.0 and 71.1, PhCH₂; 112.2, 112.4, 114.6 and 114.7, C₂ and C₆; 119.6 and 121.2, C₅; 127.2, 127.3, 127.7, 127.8, 128.2, 128.3, 128.4 and 128.6, Bn-C; 132.4 and 134.2, C₁; 144.2, 145.6 and 145.8, C₃ and C₄; 173.3, CO₂.

20

Mass spectrum: (MH⁺).

Found: *M* ; requires *M*⁺

25 **3-(3-Benzyloxy-4-hydroxyphenyl)Propionic Acid and 3-(4-Benzyloxy-3-hydroxyphenyl)Propionic Acid**

A solution of lithium hydroxide monohydrate (5.25 g, 125 mmol) in water (150 cm³) was added to a stirred solution of the mixture of methyl esters (11.95 g, 30 41.7 mmol) in THF (150 cm³). The resulting mixture was stirred under an atmosphere of nitrogen. Next morning a clear, pale yellow solution had formed. The THF was evaporated and the residue was diluted with water (150 cm³) and acidified to pH 3 with 5% HCl. The mixture was 35 extracted with CHCl₃ (3 x 350 cm³) and the combined extracts were dried (Na₂SO₄) and evaporated to a brown oil which solidified on standing. This was taken up in EtOAc

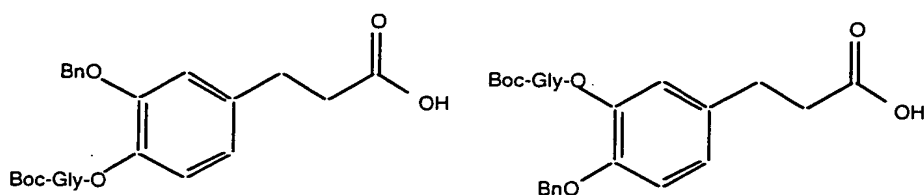
and passed through a short silica column. Evaporation of the eluent gave the product as a tan solid (11.12 g, 98%).

n_{max} (KBr disc) 3533, 3471, 3300-2600, 1718, 1699,
5 1515 cm^{-1} .

¹H NMR (300 Hz, CDCl_3) 2.66, 4H, t(*J* 7.6), CH_2CO_2 ; 2.90, 2H, t(*J* 7.6), $\text{CH}_2\text{CH}_2\text{CO}_2$; 2.91, 2H, t(*J* 7.7), $\text{CH}_2\text{CH}_2\text{CO}_2$; 5.09, 2H, s, PhCH_2 ; 5.10, 2H, s, PhCH_2 ; 6.69, 1H, dd(*J* 8.3, 2.1), H6; 6.75, 1H, dd(*J* 8.1, 2.0), H6; 6.83, 1H, d(*J* 1.9), H2; 6.84, 1H, d(*J* 2.0), H2; 6.87, 1H, d(*J* 8.4), H5; 6.90, 1H, d(*J* 8.2), H5; 7.30-7.50, 10H, m, Ar-H; CO_2H not observed.

¹³C NMR (75 MHz, CDCl_3) 29.9, 30.2, 35.7 and 35.9, 2x CH_2CH_2 ; 71.1 and 71.2, PhCH_2 ; 112.3, 112.4, 114.6 and 114.7, C2 and C6; 119.6 and 121.2, C5; 127.2, 127.3, 127.7, 127.8, 128.3, 128.4 and 128.7, Bn-C; 132.0 and 136.2, C1; 144.3, 145.6 and 145.8, C3 and C4; 179.2, CO_2 .

20 **3-(3-Benzyloxy-4-(*N*-tert-Butoxycarbonyl)glycyloxy)Phenyl-propionic Acid and 3-(4-Benzyloxy-3-(*N*-tert-Butoxycarbonyl)glycyloxy)Phenyl propionic Acid**



25

Triethylamine (1.40 cm^3 , 1.01 g, 10.0 mmol) and ethyl chloroformate (0.960 cm^3 , 1.085 g, 10.0 mmol) were added to a stirred, chilled (-20°C) solution of Boc-Gly-OH (1.75 g, 10.0 mmol) in dichloromethane (20 cm^3). The solution was stirred for 20 min at -10 - -15°C , during which time a precipitate formed. A solution of regioisomeric mixture of benzyloxyacids (2.86 g, 10.0 mmol) and triethylamine (1.40 cm^3 , 1.01 g, 10.0 mmol) in

dichloromethane (20 cm³ + 5 cm³ rinse) was then added dropwise. The resulting solution was stirred at -5 - 0°C for 2 h, then was washed with 10% citric acid (2 x 10 cm³) and brine (10 cm³), then dried (Na₂SO₄) and evaporated to a
 5 syrup. This was dissolved in a little 1:1 ethyl acetate/petroleum ether and passed through a short silica column. Evaporation of the eluent gave the mixture of
 title carboxylic acids as a colorless syrup (3.986 g, 93%).

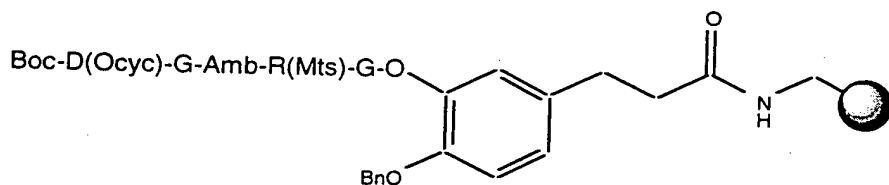
10 n_{\max} (KBr disc) cm⁻¹.

¹H NMR (300 Hz, CDCl₃) 1.47, 9H, s, CMe₃; 2.65, 2H, br t (*J* 6.6), CH₂CO₂H; 2.85-2.95, 2H, m, CH₂CH₂CO₂H; 4.15-4.17, 2H, m, NHCH₂; 5.07, 2H, s, PhCH₂O; 5.08-5.15, 1H, m, NH;
 15 6.66-7.04 and 7.29-7.46, 8H, Ar-H; CO₂H not observed.

¹³C NMR (75 MHz, CDCl₃) 28.3, CMe₃; 29.6, 30.4, 35.3 and 35.6, 2xCH₂CH₂; 42.3, NHCH₂; 70.7 and 71.3, 2xPhCH₂O; 80.1, CMe₃; 155.6, NCO₂; 178.0 and 178.4, 2xCO₂.

20

Solid-Phase Synthesis of cyclo-[D-G-Amb-R-G]



25 Aminomethyl resin (Peptide Institute, 0.83 mmol/gram, 602 mg, 0.50 mmol) was shaken with 10% DIPEA in DMF for 30, then drained and washed well with DMF.

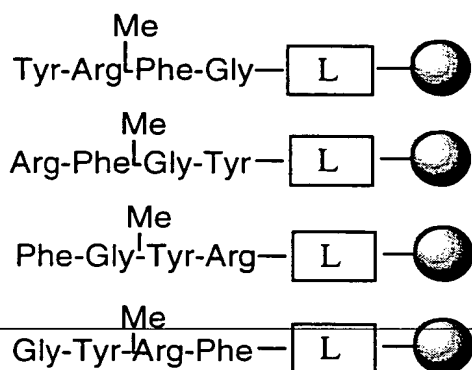
30 The benzyloxy linker (429 mg, 1.0 mmol, 2.0 equiv.) was coupled using standard HBTU/DIPEA protocols overnight. The remaining residues were coupled using standard HBTU/DIPEA protocols for ten minutes each. The final yield of the dried resin was 906 mg. Of this, 725 mg

(ca. 0.4 mmol) was cleaved with anhydrous HF using anisole as the scavenger. The resin was washed well with diethyl ether, dried at suction, then gently stirred in 5.0 cm³ DMF containing 0.5 cm³ DIPEA for 48 h. The resin was filtered
5 off and washed well with DMF. Evaporation of the filtrate, followed by preparative HPLC gave cyclo-[D-G-Amb-R-G] as a fluffy white solid (103 mg, 49%). Analysis of the product
by LC-MS indicated the presence of the cyclodimer,
cyclo-[D-G-Amb-R-G-D-G-Amb-R-G]. The ratio of monomer to
10 dimer was approximately 3:2.

Example 6 Backbone substitution and activated or safety catch linker

The backbone substitution approach (Example 1)
15 readily preorganises peptides prior to cyclisation and results in improved yields of target cyclic material. One drawback of this approach (Table 4) is increased racemisation of the C-terminus. The activated and safety catch linkers provide a means for reducing this
20 racemisation problems of the backbone substitution approach.

The array of compounds listed below is synthesised using these linkers. These compounds all contain an N-substituent, in this case a methyl group, and
25 are attached to an activated or safety catch linker. The effects of this combination on the yield and purity of the product are evaluated, paying particular attention to the degree of C-terminal racemisation.

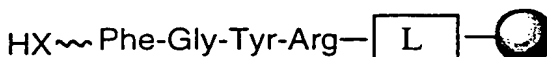


L=activated or safety catch linker

A reversible N-substituent is also used, and the effects evaluated as above.

Example 7 Ring contraction and activated or safety catch linker

5 The array of compounds listed below is synthesised using activated or safety catch linkers and ring contraction auxiliaries. The effects of this combination on the yield and purity of the product are evaluated.



HX ~ = ring contraction auxiliary;
X = O, S; L = activated or safety catch linker

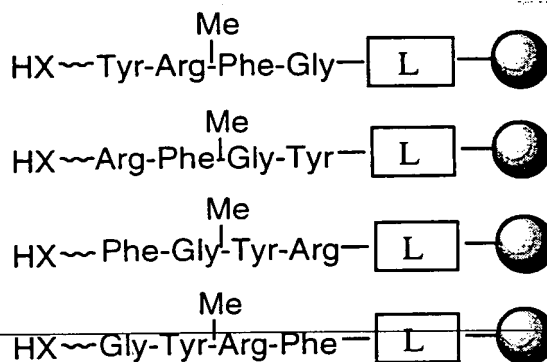
10

Example 8 Ring contraction, backbone substitution and activated or safety catch linker

15 The combination of all three approaches provides the preorganising advantages backbone substitution and ring contraction with the advantages of activated and safety catch linker cyclisation and concomitant cleavage.

20 The array of compounds listed below is synthesized using the combined approach. These compounds all contain an N-substituent, in this case a methyl group, and a ring contraction auxiliary, and are attached to an activated or safety catch linker. The effects of this combination on the yield and purity of the product are evaluated.

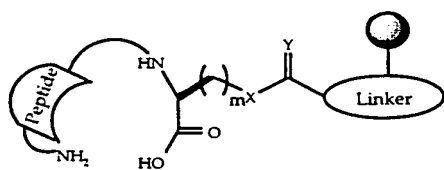
25



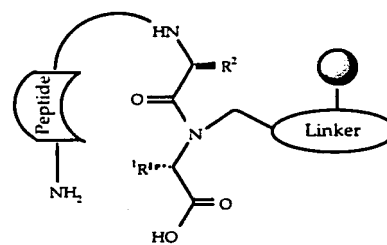
$\text{HX} \sim$ = ring contraction auxiliary;
 $\text{X} = \text{O}, \text{S}$; L = activated or
 safety catch linker

Backbone Linkers

A common approach to synthesising cyclic peptides
 5 is attachment of a C-terminal protected amino acid to the
 resin through its side chain:



Method A



Method B

10

Methodologies for peptide cyclisation on resin.

Method A - Side chain attachment

Method B - Backbone attachment

15

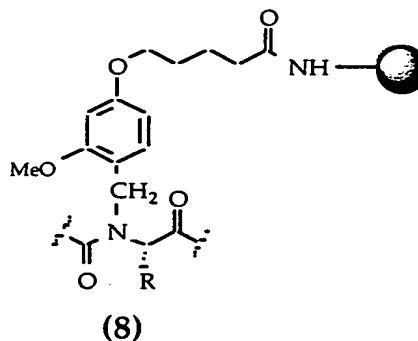
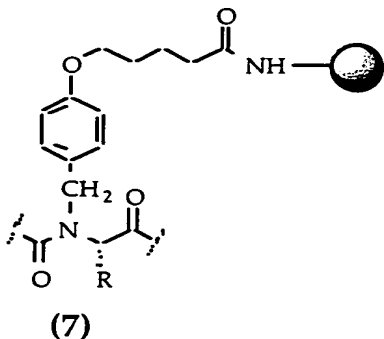
The procedure is widely applied, as it has the
 advantage of performing the cyclisation while the peptide
 is still attached to the resin, thus providing a pseudo-
 dilution environment. The cyclised peptide is then
 deprotected and cleaved to yield unprotected cyclic

20

peptide. However, from a library perspective this strategy
 is inadequate because it is restricted to the attachment of
 specific amino acids to the resin. In an attempt to

overcome these problems we have developed two backbone linkers which anchor the peptide to the resin via the first N-amide at the C-terminus.

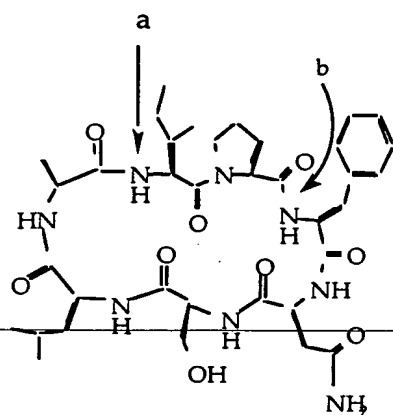
The main advantage of the backbone linking approach is that it allows flexibility in selecting the linear precursor, ie. the position of cyclisation. This is important, as yields of cyclisation are known to be dependent on the selection of the linear precursor. We have designed and developed two backbone linkers. Linker (7) permits Boc chemistry, ie. stable to neat TFA but is cleaved with HF, while linker (8) permits Fmoc chemistry, ie. is cleaved by TFA (95%):



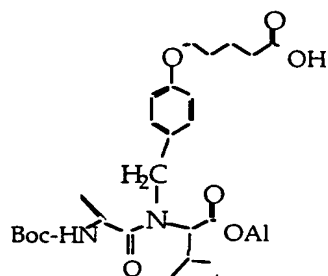
Backbone linkers investigated

Example 9 Linker (7)

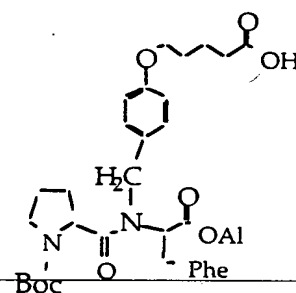
As an example we studied the synthesis of stylostatin. This cyclic heptapeptide was originally isolated from *Stylorella aurantium*, and found to be highly cytotoxic.



Stylostatin



a



b

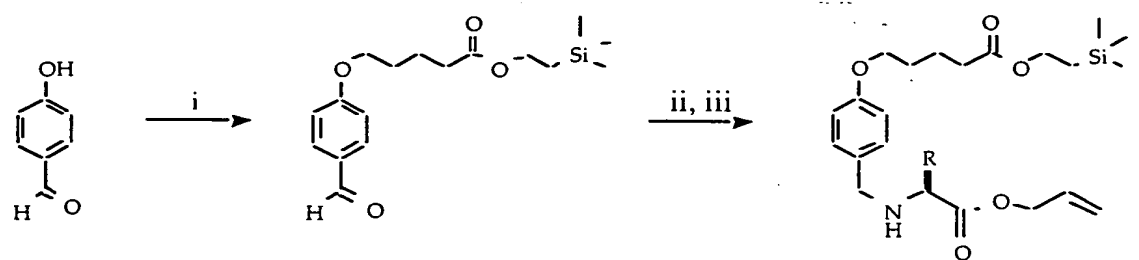
Linker-dipeptide units

The structure of stylostatin and the two linkers a and b that are used for the synthesis of stylostatin

5

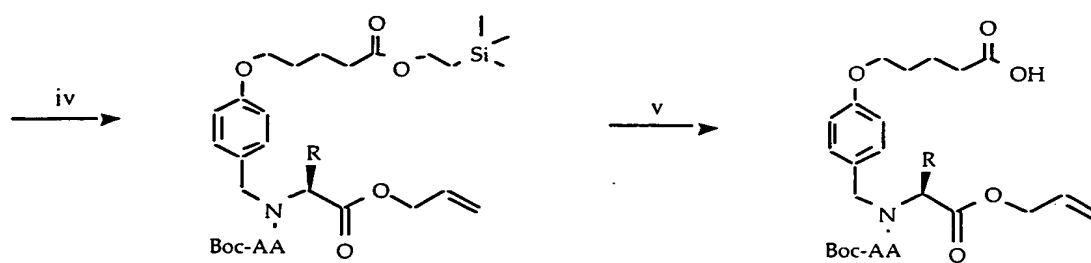
The two linker-dipeptide units, depicted above, were prepared in solution as outlined in Scheme 9, and linked to aminomethylated resin; a and b refer to the linking position on the stylostatin backbone on which the attachment to resin is made.

10



11 R = CH₂Ph

12 R = CH(CH₃)CH₂CH₃



13 R = CH₂Ph
AA = Boc-Pro

15 R = CH₂Ph
AA = Boc-Pro

14 R = CH(CH₃)CH₂CH₃
AA = Boc-Ala

16 R = CH(CH₃)CH₂CH₃
AA = Boc-Ala

Scheme 9

Reagents and Conditions:

- 5 i, BrCH₂CH₂CH₂CH₂CO₂Si(CH₃)₃, K₂CO₃, Acetone, Δ, 16 h;
- ii, H-Phe-OAllyl or H-Ile-OAllyl, MgSO₄, CH₂Cl₂, r.t., 3h;
- iii, NaBH₃CN, MeOH, r.t., 2 h;
- iv, (Boc-Pro)₂-O, DIEA, DMF, r.t., 16 h.; or
Boc-Ala-F, DIEA, THF, r.t., 30 min.;
- 10 v, TBAF, THF, r.t., 2 h.

The linear precursor sequences were then assembled on resin using *in situ* neutralisation protocols. Removal of the C-terminal allyl protection group was accomplished using Pd(Ph₃P)₄. The resin-bound linear peptide was further cyclised using BOP/DIEA activation. After deprotection and cleavage (HF), products were

separated, analysed and weighed. The reaction products consisted mainly of cyclic monomer and cyclic dimer. The results are shown in Table 6, in which the amino acid sequence is given in single-letter code.

5

Table 6

Yields of Cyclic Peptides Using Backbone Linker Approach

Resin-bound linear sequence	Backbone linking position	C-terminal	N-terminal	Yield	
				monocycle	dimer
PFNSLAI	a	Ile	Pro	25	<1
NSLAIPF	B	Phe	Asn	10	24

10

These results emphasise several interesting points. First of all, the backbone linking strategy is a feasible route towards generating cyclic peptides. The yields of isolated material, based on the substitution value of the starting resin, compare well with the overall yields obtained from solution phase cyclisation.

15

Secondly, the cyclisation yields differ significantly for the two precursors in terms of monomer versus dimer. This illustrates the advantage of the backbone linking approach over previous on-resin cyclisation approaches, ie. being

20

able to choose several precursors to the same cyclic peptide. It is generally impossible to predict the optimal precursor for cyclisation. This solid phase strategy allows one to simultaneously assemble several precursors and compare their cyclisation profiles in a fast and

25

Experimental to Example 9

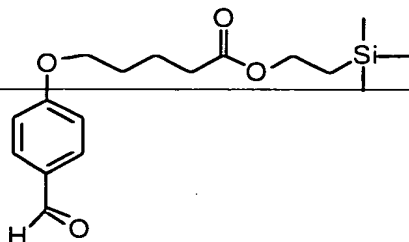
This section describes the synthetic details for the synthesis of a backbone linker and model peptides using Boc chemistry.

30

Synthesis of backbone linker (Scheme 9) and model compounds using Boc Chemistry (Table 6)

4-[5-oxy-(trimethylsilylethylvalerate)]benzaldehyde

5



$C_{17}H_{26}O_4Si$

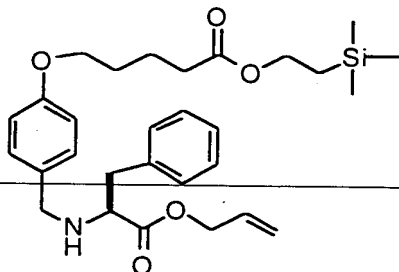
Exact Mass: 322.16

Mol. Wt.: 322.47

10

4-Hydroxybenzaldehyde (12.2 g, 0.10 mmol),
5-bromo (trimethylsilylethyl)valerate (13.82 g, 0.20 mol),
and K_2CO_3 (40.0 g, 0.29 mol) were refluxed in acetone
15 (250 mL) for 16 h. Solids were filtered, washed with
acetone and the volatiles were removed *in vacuo*. The
product was purified by column chromatography (Hexane :
EtOAc, 8:1) to yield a colourless oil (28.2 g, 87%) 1H NMR
($CDCl_3$): δ 9.87 (s, 1H, \underline{CHO}), 7.82 (d, 2H, $J = 7.0$ Hz,
Harom), 6.98 (d, 2H, $J = 7.0$ Hz, Harom), 4.20 (t, 2H,
20 $J = 6.9$ Hz, $\underline{OCH_2}$), 4.05 (t, 2 H, $J = 6.0$ Hz, $\underline{OCH_2}$),
2.42 (m, 2H, $\underline{CH_2CO}$), 1.80 (m, 4H, $\underline{CH_2CH_2}$), 0.96 (t, 2H,
 $J = 6.9$ Hz, $\underline{CH_2Si}$), 0.10 (s, 9H, $\underline{Si(CH_3)_3}$); ^{13}C NMR ($CDCl_3$) δ
190.80, 173.45, 164.026, 131.99, 131.99, 129.87, 114.72,
25 114.72, 67.82, 62.63, 34.00, 28.49, 21.55, 17.35, -1.49; MS
[$M+H$] $^+ = 323.4$ (expected 323.2).

***N*-[4-(5-oxy-(trimethylsilyl)ethylvalerate))benzyl]-L-Phenylalanine allyl ester**



5

$C_{29}N_4NO_5Si$

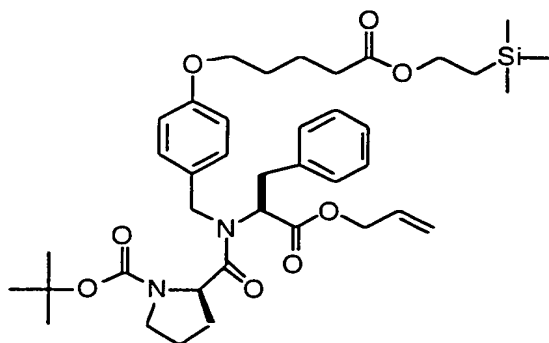
Exact Mass: 511.28

Mol. Wt.: 511.73

- The aldehyde (16.2 g, 50.2 mmol), phenylalanine allyl ester (20.5, 100 mmol) and excess $MgSO_4$ (~40 g) were stirred in CH_2Cl_2 (75 mL) at r.t. for 16 h. Solids were filtered and volatiles were removed *in vacuo* to yield the crude imine as a yellow oil. MeOH (200 mL) and HOAc (3 mL) was added and the reaction mixture was cooled to 10°C.
- NaCNBH₃ (6.1 g, 100 mmol) was added portionwise to the stirred solution. The reaction mixture was allowed to warm to room temperature before being stirred for a further 2 h. Volatiles were removed *in vacuo* and the resulting residue diluted with H₂O (100 mL) and extracted with EtOAc (3 x 100 mL). The combined EtOAc extractions were washed with saturated brine (1 x 200 mL) and water (1 x 200 mL) before being dried over $MgSO_4$. Volatiles were removed *in vacuo*, and the resulting oil purified by flash chromatography (Hexane EtOAc, 1:1) to yield a clear colourless oil (20.2 g, 79%): ¹HNMR (CDCl₃) δ 7.28 (m, 5H, H_{arom}), 7.20 (d, 2H, J = 7.0 Hz, H_{arom}), 6.85 (d, 2H, J = 7.0 Hz, H_{arom}), 5.80 (m, 1H, CH=CH₂), 5.28 (dd, 1H, J = 12.1 Hz, 1.7 Hz, CH=CH₂), 5.23 (dd, 1H, J = 10.0 Hz, 1.7 Hz, CH=CH₂), 4.55 (d, 2H, J = 6.4 Hz, PheCH₂NH₂), 4.15 (t, 2H, J = 6.9 Hz, OCH₂), 3.92 (m, 2H, OCH₂), 3.80 (dd, 2H, J = 12.2 Hz, 1.2 Hz, CH₂-CH), 3.65 (dd, 2H, J = 11.7 Hz, 1.2 Hz, CH₂-CH), 3.58 (m, 1H, CHNH), 3.05 (m, 1H, CH₂Ph), 2.25

(m, 2H, CH₂CO), 1.80 (m, 4H, CH₂CH₂), 0.95 (t, 2H, J = 6.9 Hz, CH₂Si), 0.10 (s, 9H, Si(CH₃)₃); ¹³CNMR (CDCl₃) δ 173.56, 173.00, 158.32, 136.78, 131.96, 130.67, 129.57, 129.57, 129.27, 129.27, 128.39, 128.39, 126.76, 118.77, 114.36, 114.36, 67.33, 66.48, 62.51, 61.60, 51.13, 39.18, 34.08, 28.68, 21.62, 17.32, -1.51; MS [M+H]⁺ = 512.1 (expected 512.3).

10 **Boc-L-Pro-[N-(4-(5-oxy-(trimethylsilyl)ethylvalerate))-benzyl)]-L-Phenylalanine allyl ester**



C₃₉H₅₆N₂O₈Si

Exact Mass: 708.38

Mol. Wt.: 708.96

15

Boc-Pro-OH (8.61 g, 40.0 mmol) was dissolved in EtOAc (30 mL), to which was added DCCI (4.12 g, 20.0 mmol). After activation for 10-15 min to form the symmetric anhydride, the mixture was filtered and the filtrate was added to a solution of the amine (6) (5.11 g, 10.0 mmol) and DIEA (2.67 mL, 15 mmol). The reaction was stirred at r.t. for 16 h. EtOAc (100 mL) was added and the reaction mixture was washed with 10% K₂CO₃ solution (2 x 250 mL), brine (1 x 250 mL) and H₂O (1 x 250 mL) before dried over MgSO₄. Volatiles were removed in vacuo, and the resulting oil purified by flash chromatography (Hexane : Et₂O, 5:1) to yield a clear colourless oil (3.55 g, 60%): ¹HNMR (CDCl₃) δ 7.20 (m, 7H, H_{arom}), 6.85 (d, 2H, J = 7.0 Hz, H_{arom}), 5.98 (m, 1H, CH=CH₂), 5.20 (m, 2H, CH=CH₂), 4.50 (m,

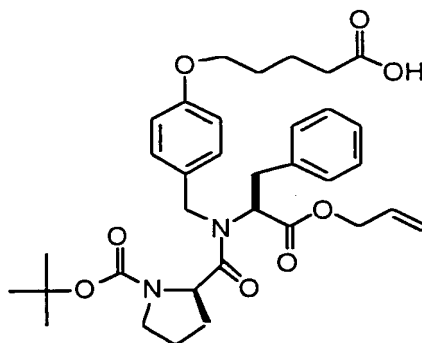
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30

3H, CH₂CH and PheCH₂N), 4.20 and 4.13 (rotomers, dd, 1H, J = 7 Hz, 2 Hz, NCH), 4.15 (t, 2H, J = 6.9 Hz, OCH₂), 3.92 (m, 2H, OCH₂), 3.71 (m, 2H, CH₂-CH), 3.31 (m, 4H, CH₂Ph and CH₂N), 2.25 (m, 2H, CH₂CO), 2.05 (m, 4H, CH₂CH₂), 1.80 (m, 4H, CH₂CH₂), 1.48 (br s, 9H, C(CH₃)₃), 0.95 (t, 2H, J = 6.9 Hz, CH₂Si), 0.10 (s, 9H, Si(CH₃)₃); ¹³CNMR (CDCl₃) δ rotomers 173.54 and 173.00, 172.42, rotomers 170.08 and 169.47, rotomers 158.68 and 158.50, rotomers 154.31 and 153.98, rotomers 138.35 and 138.05, rotomers 132.45 and 131.96, 129.40, 129.40, 129.10, 128.91, 128.63, 128.63, 127.52, rotomers 126.75 and 126.62, rotomers 118.26 and 118.06, 114.32, 114.32, rotomers 79.96 and 79.19, 67.34, rotomers 65.96 and 65.80, 62.55, rotomers 60.68 and 60.58, rotomers 57.44 and 56.94, 51.37, rotomers 46.83 and 46.77, 35.11 and 34.97, 34.07, rotomers 30.84 and 29.78, 28.67, 28.46, rotomers 24.02 and 22.77, 21.68 17.32, -1.50; MS [M+H]⁺ = 709.6 (expected 709.4).

Boc-L-Pro-[N-(4-(5-oxyvaleric acid)benzyl)]-L-Phenylalanine allyl ester



C₃₄H₄₄N₂O₈

Exact Mass: 608.31

Mol. Wt.: 608.72

25

The ester (2.0 g, 2.82 mmol) was stirred in a solution of THF (20 mL) at r.t. TBAF (3 mL, 1M) was added dropwise and saponification proceeded for 3 h. H₂O (100 mL) and HOAc (3 mL) was added to the reaction mixture.

30 The acid was extracted into EtOAc (3 x 100 mL) and was

washed H₂O (1 x 250 mL) before being dried over MgSO₄. Volatiles were removed in vacuo, and the resulting oil purified by flash chromatography (Hexane : Et₂O, 5:1) to yield a clear colourless oil. The tertiary amide

- 5 (product) was purified by column chromatography (CH₂Cl₂: MeOH, 19:1) to yield a white solid (2.54 g, 90%);

~~mp. 28-30°C : ¹H NMR (CDCl₃) δ 8.89 (br s, 1H, OH), 7.20 (m,~~
7H, H_{arom}), 6.75 (dd, 2H, J = 7.1 Hz, 1.9 Hz, H_{arom}), 5.88
(m, 1H, CH=CH₂), 5.25 (m, 2H, CH=CH₂), 4.50 (m, 3H, CH₂CH
10 and PheCH₂N), 4.20 and 4.13 (rotomers, dd, 1H, J = 6.9 Hz,
1.9 Hz, NCH), 3.88 (m, 2H, CH₂O), 3.71 (m, 2H, CH₂-CH),
3.41 (m, 4H, CH₂N, CH₂Ph), 2.25 (m, 2H, CH₂CO), 2.05-1.85
(m, 8H, 2 x CH₂CH₂), 1.48 (br s, 9H, C(CH₃)₃); ¹³C NMR (CDCl₃)
δ rotomers 179.09 and 177.04, 173.05, rotomers 170.08 and
15 169.48, rotomers 158.64 and 158.44, rotomers 154.28 and
153.96, rotomers 138.31 and 138.02, rotomers 132.43 and
131.94, 129.41, 129.41, 128.99, 128.69, 128.48, 128.48,
127.50, rotomers 126.78 and 126.65, rotomers 118.30 and
118.10, 114.37, 114.37 rotomers 80.17 and 79.38, 67.30,
20 rotomers 65.99 and 65.84, rotomers 60.72 and 60.54,
rotomers 57.49 and 57.00, 51.40, rotomers 46.86, rotomers
35.09 and 34.95, 33.56, rotomers 30.83 and 29.78, rotomers
28.46 and 20.76, rotomers 24.00 and 22.78, 21.39; MS [M+H]⁺
= 609.3 (expected 609.3).

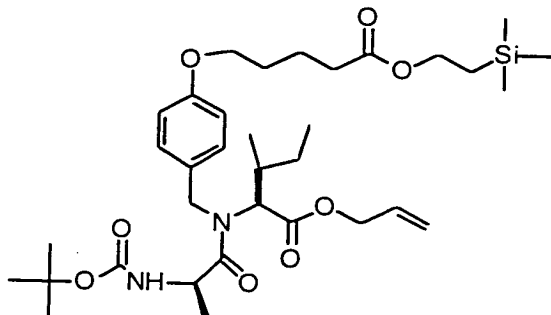
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N-[4-(5-oxy-(trimethylsilyl)ethylvalerate))benzyl]-L-Isoleucine allyl ester

The aldehyde (16.2 g, 50.2 mmol), isoleucine allyl ester (20.5, 100 mmol) and excess MgSO₄ (~40 g) were
30 stirred in CH₂Cl₂ (75 mL) at r.t. for 3 h. Solids were filtered and volatiles were removed in vacuo to yield the crude imine as a yellow oil. MeOH (200 mL) and HOAc (3 mL) was added and the reaction mixture was cooled to 10°C. NaCNBH₃ (6.1 g, 100 mmol) was added portionwise to the
35 stirred solution. The reaction mixture was allowed to warm to room temperature before being stirred for a further 2 h. Volatiles were removed in vacuo and the resulting residue

diluted with H₂O (100 mL) and extracted with EtOAc (3 x 100 mL). The combined EtOAc extractions were washed with saturated brine (1 x 200 mL) and water (1 x 200 mL) before being dried over MgSO₄. Volatiles were removed in vacuo, and the resulting oil purified by flash chromatography (1:1 hexane EtOAc) to yield a clear colourless oil (20.2 g, 79%). ¹HNMR (CDCl₃): δ 7.24 (d, 2H, J=8.0 Hz, H_{arom}), 6.85 (d, 2H, J = 8.0 Hz, H_{arom}), 5.98 (m, 1H, CH=CH₂), 5.31 (d, 1H, J = 27.2 Hz, CH=CH₂), 5.27 (dd, 1H, J = 13.2 Hz, 1.7 Hz, CH=CH₂), 5.10 (dd, 1H, J = 11.2 Hz, 1.7 Hz, CH=CH₂), 4.65 (m, 2H, PheCH₂N), 4.15 (t, 2H, J= 6.9 Hz, OCH₂), 3.92 (m, 2H, OCH₂), 3.81 (d, 1H, J = 13 Hz, CH₂-CH), 3.60 (d, 1H, J = 13 Hz, CH₂-CH), 3.17 (m, 1H, CH), 2.90 (m, CH₂CHCH₃), 2.35 (m, 2H, CHCH₂CH₃), 1.80 (m, 2H, CH₂CH₂), 1.52 (m, 1H, CHCH₂CH₃), 1.20 (m, 1H, CHCH₂CH₃), 0.95 (t, 2H, J = 6.9 Hz, CH₂Si), 0.92 (d, 3H, J = 7.6 Hz, CH₃CH), 0.90 (t, 3H, J= 7.0 Hz, CH₂CH₃), 0.10 (s, 9H, Si(CH₃)₃); ¹³CNMR (CDCl₃) δ 174.55, 174.25, 158.96 132.66, 131.22, 130.48, 130.48, 119.45, 115.02, 115.02, 68.05 65.92, 65.52, 63.20, 52.36, 38.74, 34.78, 29.39, 29.39, 26.35, 22.34, 18.02, 16.23, 12.13, -0.81; MS [M+H]⁺ = 478.3 (expected 478.3).

Boc-L-Ala-[N-(4-(5-oxy-(trimethylsilyl)ethylvalerate))-benzyl)]-L-Isoleucine allyl ester



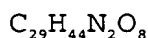
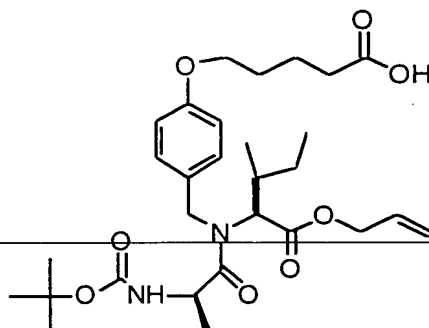
C₃₄H₅₆N₂O₈Si

Exact Mass: 648.38

Mol. Wt.: 648.90

Boc-Ala-OH (2.89 g, 15.0 mmol) was dissolved in CH_2Cl_2 (30 mL), to which was added DAST (4.12 g, 20.0 mmol). After activation for 10-15 min to form the acid fluoride, the mixture was washed with cold (H_2O ,
5 dried over MgSO_4 and the volatiles were removed *in vacuo*. The acid fluoride was then added immediately to a solution of the amine (4.78 g, 10.0 mmol) and DIEA (2.67 mL, 15 mmol) in THF (20 mL). The reaction was stirred at r.t. for 16 h. EtOAc (100 mL) was added and the reaction mixture
10 was washed with 10% K_2CO_3 solution (2 x 250 mL), brine (1 x 250 mL) and H_2O (1 x 250 mL) before being dried over MgSO_4 . Volatiles were removed *in vacuo*, and the resulting oil purified by flash chromatography (hexane : diethyl ether, 1:5) to yield a clear colourless oil (2.86 g, 44%) :
15 ^1H NMR (CDCl_3): δ 7.24 (d, 2H, $J=8.0$ Hz, H_{arom}), 6.85 (d, 2H, $J = 8.0$ Hz, H_{arom}), 5.98 (m, 1H, $\text{CH}=\text{CH}_2$), 5.31 (d, 1H, $J = 14.2$ Hz, $\text{CH}=\text{CH}_2$), 5.23 (d, 1H, $J=12.0$ Hz, $\text{CH}=\text{CH}_2$) 4.65 (m, 3H, PheCH_2N , CHCH_3), 4.15 (t, 2H, $J= 6.9$ Hz, OCH_2), 3.92 (m, 2H, OCH_2), 3.81 (d, 1H, $J = 13$ Hz, $\text{CH}_2\text{-CH}$),
20 3.60 (d, 1H, $J = 13$ Hz, $\text{CH}_2\text{-CH}$), 3.17 (m, 1H, CH), 2.90 (m, CH_2CHCH_3), 2.35 (m, 2H, CHCH_2CH_3), 1.80 (m, 2H, CH_2CH_2), 1.52 (m, 1H, CHCH_2CH_3), 1.45 (s, 9H, $\text{C}(\text{CH}_2)_3$), 1.20 (m, 1H, CHCH_2CH_3), 0.95 (t, 2H, $J = 6.9$ Hz, CH_2Si), 0.97 (s, 3H, CH_3), 0.92 (d, 3H, $J = 7.6$ Hz, CH_3CH), 0.90 (t, 3H, $J= 7.0$
25 Hz, CH_2CH_3), 0.10 (s, 9H, $\text{Si}(\text{CH}_3)_3$); MS $[\text{M}+\text{H}]^+ = 649.5$ (expected 649.4).

Boc-L-Ala-[N-(4-(5-oxyvaleric acid)benzyl)]-L-Isoleucine allyl ester



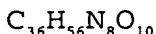
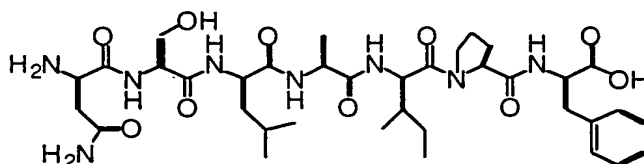
Exact Mass: 548.31

Mol. Wt.: 548.67

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The ester (2.0 g, 2.82 mmol) was stirred in a solution of THF (20 mL) at r.t. TBAF (3 mL, 1M) was added dropwise and saponification proceeded for 3 h. H₂O (100 mL) and HOAc (3 mL) was added to the reaction mixture. The acid was extracted into EtOAc (3 x 100 mL). The combined EtOAc extractions were washed with saturated brine (1 x 100 mL) and water (1 x 100 mL) before being dried over MgSO₄. Volatiles were removed *in vacuo*, and the resulting oil purified by semi-preparative HPLC (0-60% B over 60 min) to yield the tertiary amide as a colourless oil (2.54 g, 44%): ¹HNMR (CDCl₃): δ 7.22 (d, 2H, J=8.0 Hz, H_{aroma}), 6.80 (d, 2H, J = 8.0 Hz, H_{aroma}), 5.91 (m, 1H, CH=CH₂), 5.21 (d, 1H, J = 14.2 Hz, CH=CH₂), 5.22 (d, 1H, J=11.0 Hz, CH=CH₂), 4.65 (m, 3H, PheCH₂N, CHCH₃), 3.92 (m, 2H, OCH₂), 3.81 (d, 1H, J = 13 Hz, CH₂-CH), 3.60 (d, 1H, J = 13 Hz, CH₂-CH), 3.17 (m, 1H, CH), 2.90 (m, CH₂CHCH₃), 2.35 (m, 2H, CHCH₂CH₃), 1.80 (m, 2H, CH₂CH₂), 1.52 (m, 1H, CHCH₂CH₃), 1.45 (s, 9H, C(CH₃)₃), 1.20 (m, 1H, CHCH₂CH₃), 0.97 (s, 3H, CH₃), 0.92 (d, 3H, J = 7.6 Hz, CH₃CH), 0.90 (t, 3H, J=7.0 Hz, CH₂CH₃); δ MS [M+H]⁺ = 549.1 (expected 549.3).

H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH



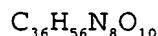
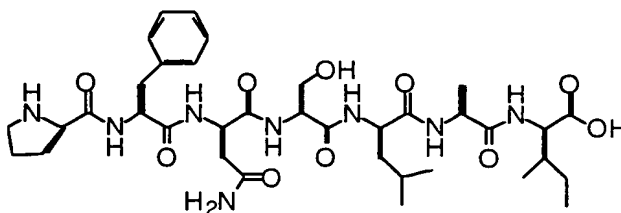
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Exact Mass: 760.41

Mol. Wt.: 760.88

The peptide was synthesised in stepwise fashion by established methods using *in situ* neutralisation/HBTU activation protocols for Boc chemistry.¹³ The Xanthyl protecting group was used for the Asn residue and the Benzyl ether for the Ser residue. Coupling reactions were monitored by quantitative ninhydrin assay and were typically >99.9%. After chain assembly was complete the removal of the allyl protecting group was achieved by the addition of tetrakis(triphenylphosphine) palladium [Pd(PPh₃)₄] (580 mg, 0.5 mmol, 3 molar equiv.) to the resin in a solution of CHCl₃ : HOAc : NMM. Vigorous shaking was initiated and continued for 14 h. The solvent was removed and the residue was washed with a 10% solution of diethyldithiocarbamic acid, sodium salt trihydrate [(C₂H₅)₂N₂CS₂Na.3H₂O] in DMF (2 x 10mL), DMF (2 x 10 mL) MeOH : CH₂Cl₂, 1: 1 (2 x 10 mL) and CH₂Cl₂ (2 x 10 mL). The N^α-Boc group removed with neat TFA (2 x 1 min treatment) and the peptide was cleaved from resin (200 mg, 0.166 mmol/g) using HF : p-cresol, 11 mL, 10:1, for 1 h at -5°C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether before being dissolved in the HPLC buffer and lyophilized. The peptide H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH (20) was purified by semi-preparative HPLC (30-90% B over 60 min) to yield a white powder (25 mg 78%); MS [M+H]⁺ = 761.21 (expected 761.42)

H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH



Exact Mass: 760.41

Mol. Wt.: 760.88

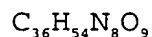
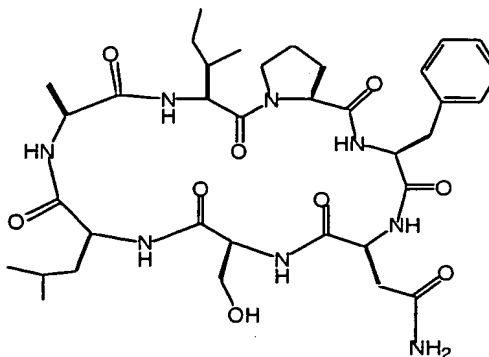
5

The peptide was synthesised using a similar procedure to that in the previous experiment above using the precursor Boc-Ala-[Backbone attachment]-Ile-O-Allyl (200 mg, 0.180 mmol/g). The peptide H-Pro-Phe-Asn-Ser-Leu-Ala-Ile was purified by semi-preparative HPLC (30-90% B over 60 min) to yield a white powder (10.5 mg, 39%); MS $[M+H]^+ = 761.2$ (expected 761.4).

15

Solution Cyclization

Method 1: Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile)



Exact Mass: 742.40

Mol. Wt.: 742.86

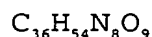
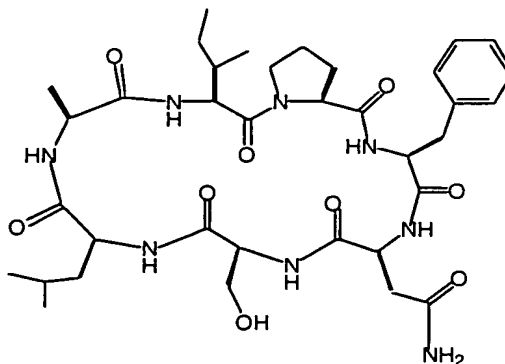
20

The linear peptide H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH (15.0 mg, 0.020 mmol) and BOP (26.1 mg, 0.060 mmol) was

25

stirred in DMF (19.7 mL, 1×10^{-3} M) at -10°C . DIPEA (35 μL , 0.197 mmol) was added dropwise to the solution. After the reaction was left to stir for a further 2 h at this temperature, all volatiles were removed *in vacuo*. The peptide Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) was purified by semi-preparative HPLC (30-90% B over 60 min) to yield a white powder (7.0 mg, 48%). $^1\text{H NMR}$ (DMSO): δ MS $[\text{M}+\text{H}]^+ = 743.2$ (expected 743.4092). Also isolated was the dimer, Cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (3 mg, 21%); MS $[\text{M}+\text{H}]^+ = 1486.2$ (expected 1486.8), and the trimer, Cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (0.7 mg, 5%); MS $[\text{M}+\text{H}]^{2+} = 1115.1$ (expected 1115.1)

15 **Method 2: Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile)**



Exact Mass: 742.40

Mol. Wt.: 742.86

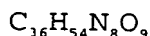
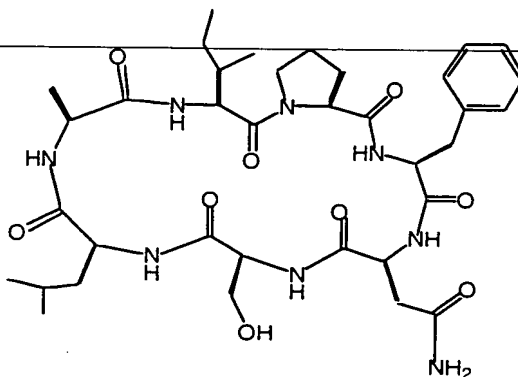
20

The peptide was synthesized using a similar procedure to Method 1 above using H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH (100 mg, 0.131 mmol), BOP (174 mg, 0.393 mmol), and DIPEA (228 μL , 1.31 mmol). The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) was purified by semi-preparative HPLC (10-70% B over 60 min) to yield a white powder (10.5 mg, 67%); MS $[\text{M}+\text{H}]^+ = 743.2$ (expected 743.4092). All other physical characteristics (^1H NMR, m.p., HPLC retention

time, and amino acid analysis) were also consistent with the results reported for Method 1.

On-Resin Cyclization

5 **Method 1: Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile)**



Exact Mass: 742.40

Mol. Wt.: 742.86

10

After chain assembly for the linear peptide was complete (synthesised from the solid support where the linker was attached between Boc-Pro-Phe-O-Allyl). The allyl protecting group and the N^α-Boc group was removed with [Pd(PPh₃)₄] (580 mg, 0.5 mmol) and TFA (2 x 1 min treatment) the reaction mixture was then cooled to -10°C and BOP (221 mg, 0.5 mmol) was added. 2,6 Lutidine (194 µL, 1.66 mmol) was then added dropwise and the reaction continued until the ninhydrin assay found an absence of amine <0.1%. The organic material was filtered from the resin (250 mg, 0.167 mmol/g) and the cyclic peptide was cleaved from resin using HF : p-cresol, 11 mL, 10:1, for 1 h at -5°C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether before being dissolved in the HPLC buffer and lyophilized. The peptide Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) was purified by semi-preparative HPLC (30-90% B over 60 min) to yield a white powder (3.1 mg, 10%): ¹HNMR (DMSO)

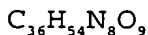
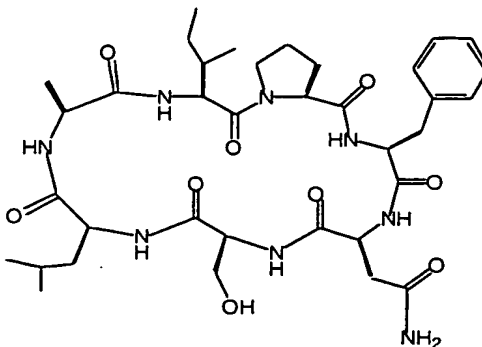
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20

25

δ MS $[M+H]^+ = 743.2$ (expected 743.4092). Also isolated was the dimer, Cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (7.6 mg, 24.5%); MS $[M+H]^+ = 1486.2$ (expected 1486.8), and the trimer, Cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (0.4 mg, 1%); MS $[M+H]^{2+} = 1115.2$ (expected 1115.1). All other physical characteristics (1H NMR, m.p., HPLC retention time, and amino acid analysis) were also consistent with what was reported above.

Method 2: Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile)



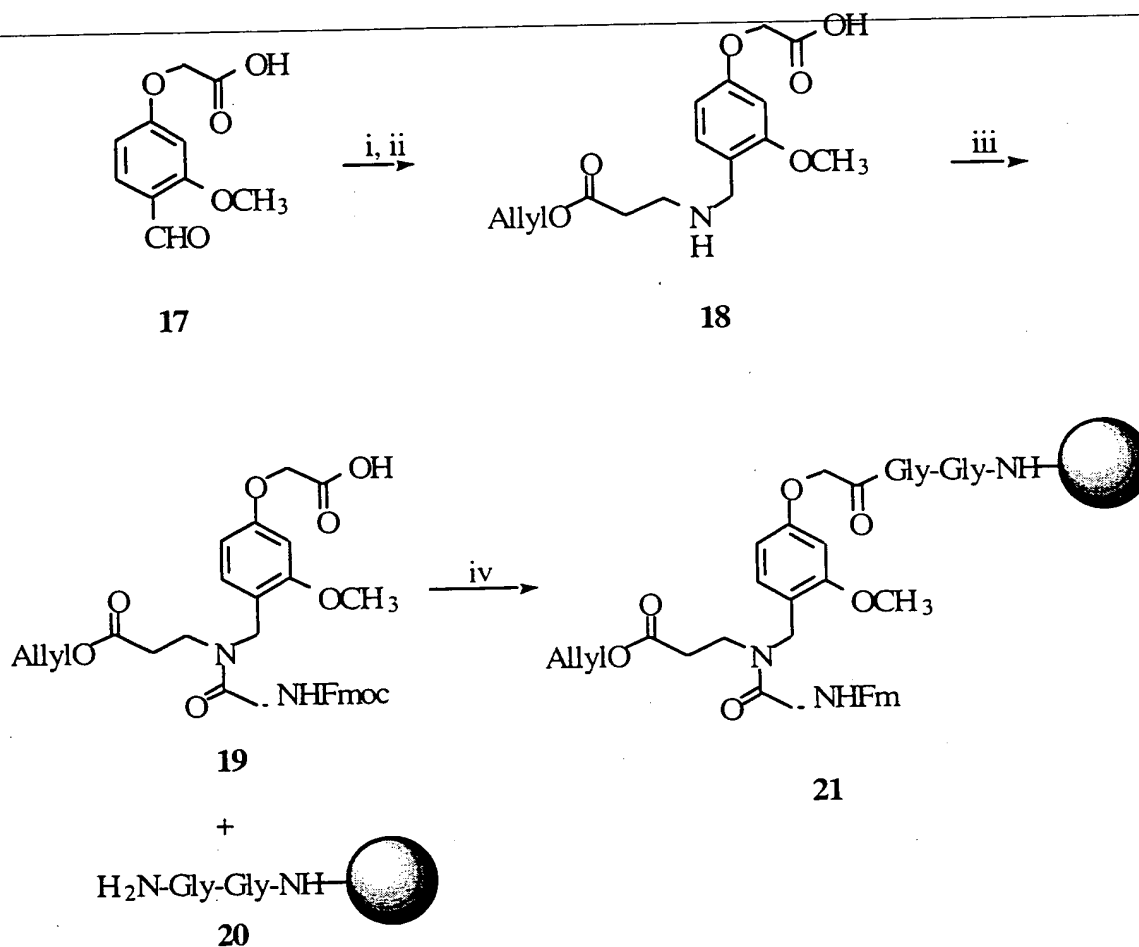
Exact Mass: 742.40

Mol. Wt.: 742.86

The peptide was synthesized using a similar procedure to Method 1 using the precursor where the linker was attached between Boc-Ala-Ile-O-Allyl (200 mg, 0.203 mmol/g), $[Pd(PPh_3)_4]$ (290 mg, 0.250 mmol), BOP (60 mg, 0.136 mmol), and 2,6-lutidine (?? μ L, 2.03 mmol). The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) (3) was purified by semi-preparative HPLC (30-90% B over 60 min) to yield a white powder (8.2 mg, 25%); MS $[M+H]^+ = 743.2$ (expected 743.4). All other physical characteristics (1H NMR, m.p., HPLC retention time, and amino acid analysis) were also consistent with what was reported above.

Example 10 Fmoc-Based Synthesis Using Linker 8

Similar to linker (7), we have employed linker (8) for the Fmoc-based synthesis of a series of cyclic pentapeptides. The synthesis of the linker is illustrated in Scheme 10, and cyclic products obtained using this linker are listed in Table 7.



- 10 i, $\text{NH}_2(\text{CH}_2)_2\text{CO}_2\text{-Allyl}$, MgCl_2 , THF, r.t. 72 h;
 ii, NaCNBH_3 , CH_3OH , r.t., 2 h;
 iii BOP, Fmoc-Gly-OH, DIEA, DMF, 24 h;
 iv HBTU, DIEA, DMF, 120 min

15

Scheme 10
Reagents and Conditions

Table 7

Cyclisation Yields Using Fmoc Backbone Linker

Peptide Sequence	Yield (%)	Reaction Time
cyclo-[Leu-Asp-Val-Gly- β -Ala]	18%	12 h
cyclo-[Arg-Gly-Asp-Gly- β -Ala]	9%	24 h
cyclo-[Phe-Lys-Trp-Gly- β -Ala]	15%	12 h

5

Experimental to Example 10

This section describes the synthetic details for the synthesis of a backbone linker and model peptides using Fmoc chemistry.

10

Synthesis Of Backbone Linker And Model Compounds using Fmoc Chemistry

General Methods

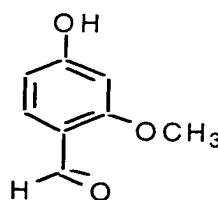
15 The fluorenyl-protected amino acids were coupled onto the resin as their free acids (4 mol equiv.) by addition of HBTU (4 mol equiv.) and DIEA (5 mol. equiv.). The couplings were performed in DMF for 20 min. After each successive coupling the resin was rinsed successively with DMF, MeOH and DCM before monitoring the success with
20 Kaiser ninhydrin assay. Removal of the Fmoc group was achieved by treatment (10 min) with 20% piperidine in DMF. Removal of the allyl protecting group was achieved by the addition of Pd(PPh₃)₄ (3 mol equiv.) to the resin in a solution of CHCl₃ : HOAc : NMM, 37:2:1, 5 mL under an
25 atmosphere of nitrogen. Shaking was initiated and continued for 3 h. The resin was rinsed successively with a solution of 10% sodium dithiodicarbonate trihydrate in DMF (twice), DMF, MeOH and DCM, and dried in vacuo.

30 Linear peptides were removed by TFA (100%) 5 h and checked for purity by HPLC. HPLC was carried out on a Waters apparatus at λ =254 nm on an analytical Vydac column using an isocratic elution with 70% buffer A (H₂O, 0.1%

TFA) for 5 minutes, followed by a 2.5% linear gradient to 80% buffer B (90% CH₃CN, 10% H₂O, 0.1% TFA) at 2 mL/min flow rate. After the final removal of the Fmoc group, the resin was rinsed with DMF before HATU (5 mol equiv.) was added portionwise to the resin in a solution of DMF (2 mL). DIEA (10 mol equiv.) was added dropwise and shaking was initiated and continued for 6 h before a further 5 mol. equiv. HATU and 10 mol. equiv. DIEA was added. Shaking was again recontinued until the resin gave a negative ninhydrin test. The resin was rinsed once again with DMF, MeOH and DCM, and dried in vacuo.

Cyclic peptides were removed by TFA (100%) 5 h and purified by HPLC. HPLC was carried out on a Waters apparatus at $\lambda=214$ nm on a semi-preparative Vydac column using an isocratic elution with 100% buffer A (H₂O, 0.1% TFA) for 10 minutes, followed by a 1% linear gradient to 50% buffer B (90% CH₃CN, 10% H₂O, 0.1% TFA) at 10 mL/min flow rate.

20 **3-Methoxy-4-formylphenol (3)**



Exact Mass: 152.05

Mol. Wt.: 152.15

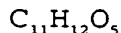
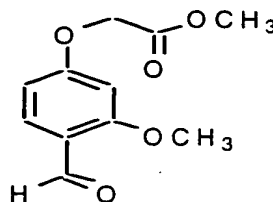
25

30 In a 1 L three-necked flask fitted with a dropping funnel, thermometer and drying tube was placed 3-methoxyphenol 5 (70 g, 0.64 mol) and freshly distilled phosphoryl chloride (100 mL, 1.08 mol). The solution was stirred at 0°C whilst DMF (75 mL, 0.97 mol) was added dropwise over 45 min. The solution was further stirred for 24 h before the pale oil was poured onto crushed ice (1 L)

and after 10 min the cloudy solution was washed with ether (2 x 300 mL). The aqueous layer was once again cooled to 0°C and adjusted to pH 5.5-6 by careful addition of NaOH (39 g, 0.98 mol) and then NaOAc (380 g, 4.63 mol). Water (150 mL) and ethyl acetate (EtOAc) (500 mL) were added, and the aqueous layer was washed further with EtOAc (250 mL).

~~The combined organic extracts were washed with brine~~ (250 mL) and water (250 mL), dried over MgSO₄, and evaporated. The residue was triturated with boiling petroleum spirit and the crystalline solid was collected to give the title compound (25.2 g, 27.2%), m.p. 154-5°C [lit m.p.¹² 158.5-160°C]; δ_{H} (d⁶-acetone) 3.08 (1H, br s, OH), 4.92 (2H, s, OCH₃), 6.54 (1H, dd, J 9 Hz, J 2 Hz, 6^{Ar}-H), 6.57 (1H, d, J 2 Hz, 2^{Ar}-H), 7.77 (1H, d, J 9 Hz, 5^{Ar}-H), 10.24 (1H, s, CHO); δ_{C} (d⁶-acetone) 52.76, 99.27, 108.76, 118.63, 130.32, 164.73, 165.29, 187.07.

Methyl 3-methoxy-4-formylphenoxy ethyl ester



Exact Mass: 224.07

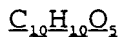
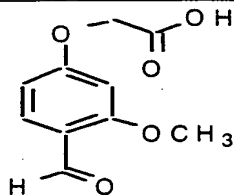
Mol. Wt.: 224.21

In a 500 mL flask were added the phenol (24 g, 0.166 mol), methyl bromoacetate (75 g, 0.49 mol) and K₂CO₃ (67.0 g, 0.49 mol) in acetone (100 mL). The reaction mixture was stirred at reflux for 16 h, cooled to room temperature, filtered, and evaporated under reduced pressure. The oily residue was purified by flash column chromatography EtOAc:Hexane (1:3), to give the methyl ester (31.63 g, 85%), m.p. 79-81°C; δ_{H} (CDCl₃) 3.82 (3H, s, OCH₃), 4.82 (2H, s, OCH₂), 4.80 (2H, s, CH₂), 6.48 (1H, dd, J 9 Hz,

\underline{J} 2 Hz, 6^{Ar} -H), 6.57 (1 H, d, \underline{J} 2 Hz, 2^{Ar} -H), 7.80 (1 H, d, \underline{J} 9 Hz, 5^{Ar} -H), 10.29 (1H, s, CHO); δ_C (CDCl₃) 52.45, 55.68, 65.07, 99.24, 105.40, 119.84, 130.76, 163.48, 163.96, 168.46, 188.27.

5

3-Methoxy-4-formylphenoxy acetic acid



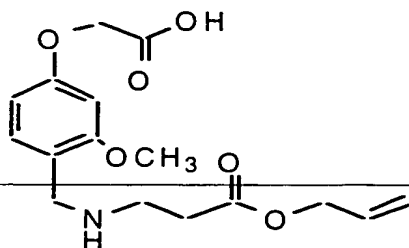
10

Exact Mass: 210.05

Mol. Wt.: 210.18

LiOH (0.5 M, 75 mL) was added dropwise to a stirred solution of the methyl ester (7.5 g, 33.45 mmol) in H₂O:THF, 3:2 (100 mL) at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for a further 16 h. EtOAc (250 mL) and a Citric acid solution (20%, 500 mL) was added, and the aqueous layer was washed with EtOAc (250 mL). The combined organic extracts were then washed with brine (250 mL) and water (250 mL), dried over MgSO₄, and evaporated to dryness under reduced pressure to give the title compound (6.75 g, 96%), m.p. 106-7°C [lit m.p.¹² 106-7°C]; δ_H (d⁶-acetone) 3.40 (1H, s, OH), 3.82 (3H, s, OCH₃), 3.92 (3H, s, OCH₃), 6.48 (1H, dd, \underline{J} 9 Hz, \underline{J} 2 Hz, 6^{Ar} -H), 6.57 (1 H, d, \underline{J} 2 Hz, 2^{Ar} -H), 7.80 (1 H, d, \underline{J} 9 Hz, 5^{Ar} -H), 10.29 (1H, s, CHO); δ_C (d⁶-acetone) 56.06, 99.01, 106.93, 118.49, 129.80, 163.32, 164.49, 169.57, 187.27.

Allyl 3-amino-[methyl-(2'-methoxy-4'-phenoxy acetic acid)]
propanoic ester



5

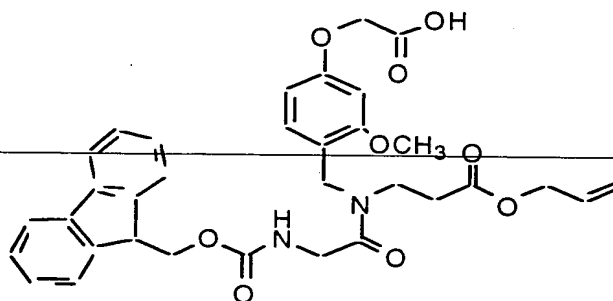
$C_{16}H_{21}NO_6$

Exact Mass: 323.14

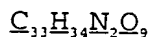
Mol. Wt.: 323.34

The aldehyde (1.87 g, 8.92 mmol) and the amine
10 (2.58 g, 20 mmol) was stirred at room temperature in THF
(40 mL) in the presence of dry $MgSO_4$ (15 g) for 72 h. The
reaction mixture was filtered, and evaporated to dryness
under reduced pressure to give a solid residue. The solid
was then dissolved in methanol (MeOH) (50 mL) and $NaCNBH_3$
15 was added portionwise over 10 minutes. The reaction
mixture was allowed to stir for a further 3 h before ether
(100 mL) was added. The amino acid was extracted into H_2O
(3 x 250 mL). Excess NaCl was then added to the H_2O layer
and the amino acid was extracted back into EtOAc
20 (3 x 100 mL). The combined organic layers were dried over
 $MgSO_4$, and evaporated to dryness under reduced pressure to
give the title compound as an unpurified oil (2.59 g, 90%);
 δ_H (d^6 -acetone) 2.95 (2H, t, J 7 Hz, CH_2NH), 3.40 (2H, m,
 CH_2CO), 3.89 (3H, s, OCH_3), 4.22 (2H, m, CH_2O), 4.42 (2H,
25 s, OCH_2), 5.23 (2H, dd, J 24, J 10 Hz, $CH=CH_2$), 5.91 (1H,
m, CH), 6.58 (1H, dd, J 9 Hz, J 2 Hz, 6^{Ar} -H), 6.68 (1 H,
d, J 2 Hz, 2^{Ar} -H), 7.42 (1 H, d, J 9 Hz, 5^{Ar} -H), 8.85 (1H,
s, OH); δ_C (d^6 -acetone) 43.27, 47.61, 50.10, 64.49, 66.14,
99.82, 106.30, 112.67, 118.43, 132.97, 133.29, 160.00,
30 161.67, 170.58, 171.40.

Allyl 3-amino-[carboxymethyl-N-(9'-fluorenylmethoxy-carbonyl)-amino]-[methyl-(2'-methoxy-4'-phenoxy acetic acid)] propanoic ester



5



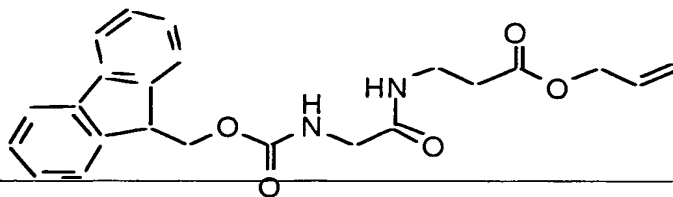
Exact Mass: 602.23

Mol. Wt.: 602.63

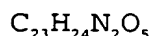
10 The amino acid (518 mg, 1.6 mmol) was added
portionwise to a stirred solution of Fm-Gly-OH (594 mg,
2 mmol), BOP (884 mg, 2 mmol) and DIEA (1 mL) in DMF (5 mL)
at r.t. The reaction mixture was allowed to stir for a
further 24 h, before being evaporated to dryness under
15 reduced pressure. EtOAc (50 mL) and Citric Acid (10%,
50 mL) were added, and the aqueous layer was washed further
with EtOAc (50 mL). The combined organic extratcts was
washed with brine (50 mL) and water (50 mL), dried over
MgSO₄, and evaporated to dryness under reduced pressure.
20 The title compound was purified by HPLC (C-18 reverse
phase). HPLC was carried out at $\lambda=254$ nM on a Vydac column
using a 1.0% linear gradient from 70% buffer A (H₂O, 0.1%
TFA) to 80% buffer B (90% CH₃CN, 10% H₂O, 0.1% TFA) at
20 ml/min flow rate (522 mg, 53%).

25

Cleavage of Fmoc-Gly- β -Ala-O-Allyl from the Acid-Labile Linker



5



Exact Mass: 408.17

Mol. Wt. 408.45

10 Cleavage was performed with 5 mg of the tertiary
amide being stirred in TFA (2 mL) for 5 h. The mixture was
evaporated to dryness. HPLC was carried out at $\lambda=254$ nm on
an analytical Vydac column using an isocratic elution 70%
15 buffer A (H_2O , 0.1% TFA) for 5 minutes followed by a 2.5%
linear gradient from to 80% buffer B (90% CH_3CN , 10% H_2O ,
0.1% TFA) at 10 ml/min flow rate. The dipeptide co-eluted
with the known sample and gave the correct molecular ion.

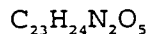
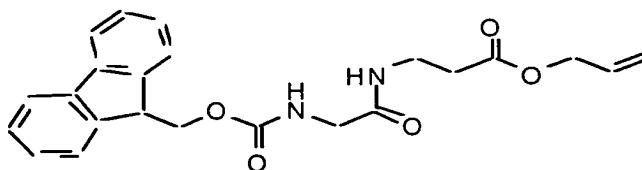
Procedure for the Attachment of the Acid Labile Linker to the Solid Support

20 DIEA (0.49 mL, 2.75 mmol) was added to a solution
of Boc-Gly-OH (43.75 mg, 0.25 mmol), and HBTU (95 mg,
0.25 mmol) in DMF (4mL). This mixture was then added to
Aminomethyl Polystyrene Resin (0.83 mmol/g, 1.0 g).
Shaking was initiated and continued for 20 min before being
25 rinsed with DMF. Pyridine : DMF : Acetic anhydride (Ac_2O)
(1:1:8, 5 mL) was then added and shaking was recontinued
for a further 20 min before being rinsed with excessive
amounts of DMF. Removal of the Boc group was achieved by
treatment with TFA (2 x 1 min). A second Boc-Gly-OH
30 (175 mg, 1.0 mmol) was attached by a similar method [DIEA
(0.49 mL, 2.75 mmol), HBTU (379 mg, 1.0 mmol) in DMF
(4mL)]. Once again removal of the Boc group was achieved
by treatment with TFA (2 x 1 min). Attachment of Allyl

3-amino-[carboxymethyl-N-(9'-fluorenylmethoxycarbonyl)-
amino] - [methyl-(2'-methoxy-4'-phenoxy acetic acid)]
propanoic ester **8** was achieved by the addition of the acid
(301 mg, 0.5 mmol), DIEA (0.27 mL, 1.5 mmol) HBTU (180 mg,
5 0.5 mmol) in DMF (4mL)] to the resin. Shaking was
initiated and continued for 20 min before being rinsed with
DMF, MeOH and dichloromethane (DCM), and dried in vacuo.
After each coupling onto the resin the success of coupling
was monitored with Kaiser ninhydrin assay.

10

Cleavage of Fmoc-Gly- β -Ala-O-Allyl from solid support



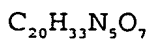
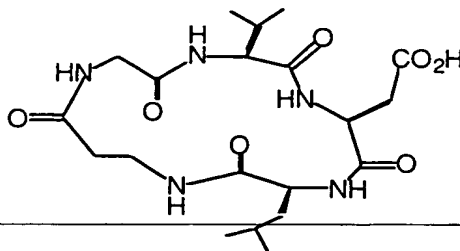
15

Exact Mass: 408.17

Mol. Wt.: 408.45

Cleavage was performed with 10 mg of resin being
stirred in TFA (2mL) for 5 h. The mixture was evaporated
20 to dryness under reduced pressure before being taken up in
a solution of H₂O : CH₃CN, (1:1, 5 mL), filtered and then
lyophilised. HPLC was carried out at λ =254 nm on a semi-
preparative Vydac column using an isocratic elution 90%
buffer A (H₂O, 0.1% TFA) for 10 minutes followed by a 1.0%
25 linear gradient from to 70% buffer B (90% CH₃CN, 10% H₂O,
0.1% TFA) at 10 ml/min flow rate.

Cyclo-[Leu-Asp-Val-Gly-β-Ala]



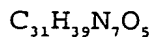
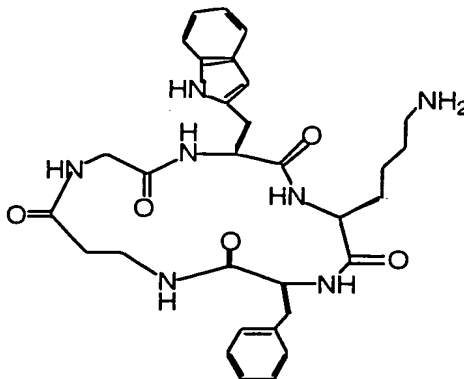
Exact Mass: 455.24

Mol. Wt.: 455.51

5

Cyclo-[Leu-Asp-Val-Gly-β-Ala] was lyophilised to a white powder (12.3 mg, 18%): MS $[M+H]^+ = 456.3$ (456.3);
10 Amino Acid Analysis: Gly = 1.06, β-Ala = 1.01, Asp = 1.03, Val = 1.03, Leu = 0.88.

Cyclo-[Phe-Trp-Lys-Gly-β-Ala]



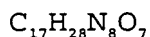
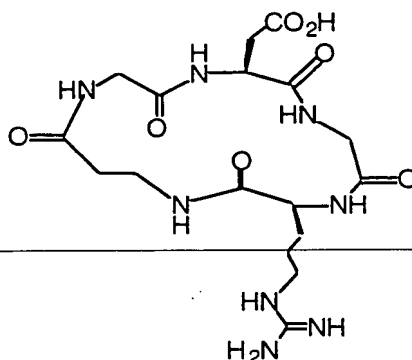
Exact Mass: 589.30

Mol. Wt.: 589.69

15

Cyclo-[Phe-Trp-Lys-Gly-β-Ala] was lyophilised to a white powder (8.1 mg, 9%): MS $[M+H]^+ = 590.1$ (expected 590.3). Amino Acid Analysis: Gly = 0.99, β-Ala = 1.01, Lys = 1.04, Phe = 1.02, Trp = 0.95.
20

Cyclo-[Arg-Gly-Asp-Gly-β-Ala]



Excat Mass: 456.21

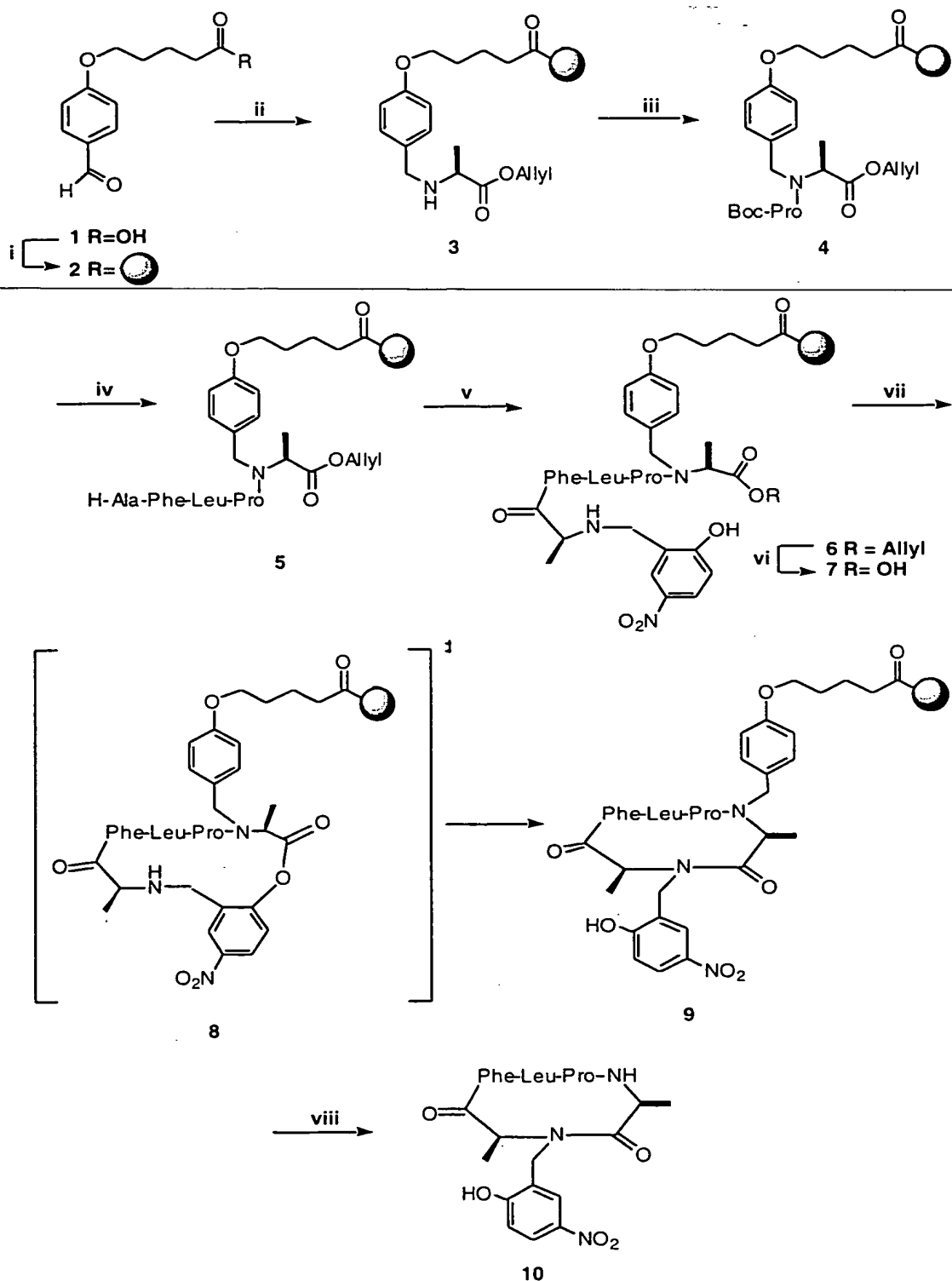
Mol. Wt.: 456.45

Cyclo-[Arg-Gly-Asp-Gly-β-Ala] was lyophilised to a white powder (8.2 mg, 15%): MS $[M+H]^+ = 457.1$ (457.3).

Amino Acid Analysis: Gly = 1.95, β-Ala = 1.01, Asp = 0.96, Arg = 1.09.

Example 11 Backbone Linker Plus Ring Contraction

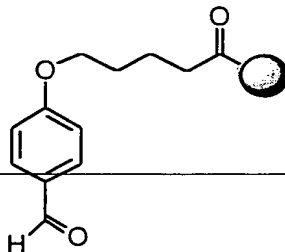
As is emphasised below, we have evaluated the combination of the backbone linker and ring contraction approach in the synthesis of cyclo [Ala Pro Leu Phe Ala]. In this instance the peptide was assembled on the backbone linker, and the ring contraction auxiliary appended to the N-terminus through reductive amination. Initial cyclisation and ring contraction were allowed to proceed on resin. The resulting cyclic product was then cleaved off the resin using anhydrous HF.



^aReagents: i, H-Gly-Leu-Leu-, HBTU, DIEA, DMF, r.t.; ii, Ala-OAllyl, NaBH₃CN, 5% HOAc/MeOH, r.t., 3 h; iv, (Boc-Pro)₂-O, DCM, r.t., 16 h; iv, SPPS; v, 2-Hydroxy-4-nitro-benzaldehyde, NaBH₄, DMF, 2 h; vi, Pd(Ph₃)₄, CH₃Cl : HOAc : NMM, 37:2:1, r.t., 3 h; vii DIC, DIEA, 70 °C, 2 h; viii, HF : p-cresol, 10:1, -5 °C, 1 h

Experimental to Example 11

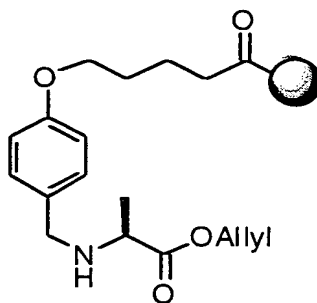
4-(5-Oxyvaleric acid)benzylaldehyde appended to resin 2



5

4-(5-Oxyvaleric acid)benzylaldehyde **1** (0.89 g, 4.0 mmol) and HBTU (1.52 g, 4.0 mmol) was dissolved in DMF (10 mL). DIEA (1 mL) was added to the solution, and this reaction mixture was then added to the precoupled H-Gly-Leu-Leu-aminomethylpolystyrene resin. Substitution value of aminomethylpolystyrene resin (4.8 g, sv=0.21 mmol/g). Shaking was continued for 30 minutes, the eluant filtered off and the resin was washed with DMF (2 x 10 mL), CH₂Cl₂ : MeOH (1: 1, 2 x 10 mL) and CH₂Cl₂ (2 x 10 mL) before being dried.

N-[4-(5-oxyvaleric acid)benzyl]-L-Alanine allyl ester appended to resin **3**

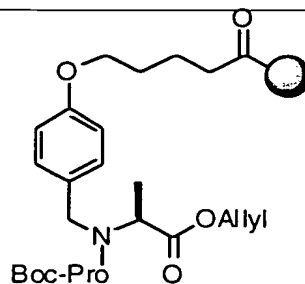


20

The aldehyde **2** and alanine allyl ester (1.29 g, 10 mmol) was dissolved in 5% HOAc/MeOH (10 mL). The reaction mixture was stirred at room temperature for 5 min before NaBH₃CN (0.61 g, 10 mmol) was added portionwise to the solution. The reaction mixture was allowed to stir for a further 2 h before the eluant was filtered off. The

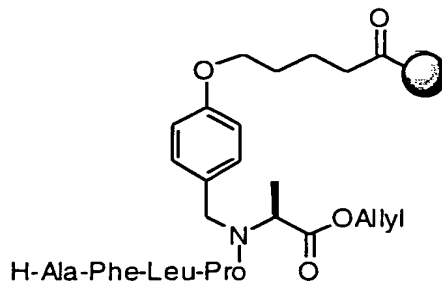
resin was washed with 5% HOAc/MeOH (2 x 10 mL), 5% DIEA/MeOH (3 x 10 mL), CH₂Cl₂ : MeOH (1: 1, 2 x 10 mL) and CH₂Cl₂ (2 x 10 mL) before being dried.

- 5 *Boc-Pro-[N-(4-(5-oxyvaleric acid)benzyl)]-L-Alanine allyl ester appended to resin 4*



- 10 Boc-Pro-OH (4.31 g, 20.0 mmol) was dissolved in CH₂Cl₂ (10 mL), to which was added diisopropylcarbodiimide DIC (1.26 g, 10.0 mmol). After activation for 10-15 min to form the symmetric anhydride, the mixture was filtered and the filtrate was added to the resin 3. The reaction was
15 shaken at r.t. for 16 h before the eluant was filtered off. The resin was washed with CH₂Cl₂ (5 x 10 mL) before being dried.

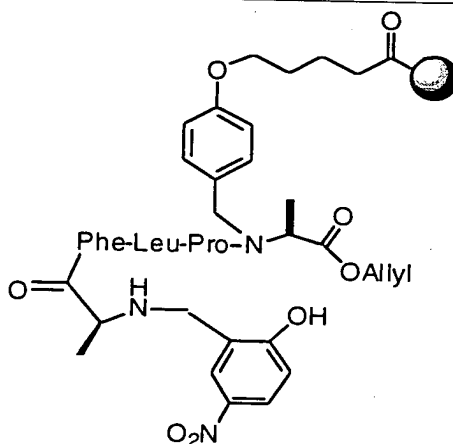
- 20 *H-Ala-Phe-Leu-Pro-[N-(4-(5-oxyvaleric acid)benzyl)]-L-Alanine allyl ester appended to resin 5*



- 25 The peptide 5 was synthesised in stepwise fashion by established methods using *in situ* neutralisation/HBtU activation protocols for Boc chemistry. Coupling reactions

were monitored by quantitative ninhydrin assay, and were typically >99.9%.

5 *N*-(2-hydroxy-4-nitrobenzyl)-Ala-Phe-Leu-Pro-[*N*-(4-(5-oxyvaleric acid)benzyl)]-L-Alanine allyl ester appended to resin 6



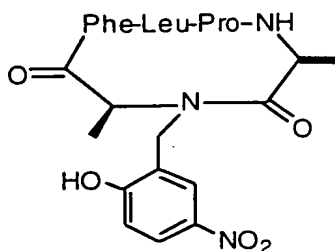
10 2-Hydroxy 4-nitro-benzaldehyde (1.67 g, 10 mmol) and the peptide on resin 5 was stirred in DMF (4 mL) at r.t. for 5 min. NaBH₄ (0.34 g, 10 mmol) was added portionwise to the solution, and the reaction mixture allowed to stir for a further 1 h before the eluant was
15 filtered off. The addition of the benzaldehyde and NaBH₄ in DMF (10 mL) was then repeated once. The resin was washed with DMF (3 x 10 mL), CH₂Cl₂ : MeOH (1:1, 2 x 10 mL) and CH₂Cl₂ (2 x 10 mL) before being dried.

20 The allyl protecting group was achieved by the addition of tetrakis(triphenylphosphine) palladium [Pd(PPh₃)₄] (1.74 g, 0.5 mmol) to the resin in a solution of CHCl₃:HOAc:NMM (37:2:1) and continued stirring for 14 h. The solvent was removed and the residue was washed with a 10% solution of diethyldithiocarbamic acid (sodium salt trihydrate [(C₂H₅)₂N₂CS₂Na.3H₂O]) in DMF (2 x 10 mL), then
25 with DMF (2 x 10 mL), MeOH : CH₂Cl₂ 1: 1 (2 x 10 mL) and finally with CH₂Cl₂ (2 x 10 mL).

A small amount of the peptide 7 was cleaved from the resin (100 mg, 0.166 mmol/g) using HF:p-cresol, 5.5 mL, 10:1, for 1 h at -5°C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether, filtered, dissolved in the HPLC buffer and lyophilized. Analytical HPLC (20-70% B over 20 min) showed only one peak; ES-MS M_r 668.4 (calcd 669.3).

Cyclo-[N-(2-hydroxy-4-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala] 10

10



DIC (6.7 mg, 0.04 mmol) was added to a solution of the peptide on resin 7 (200 mg, sv = 0.176 mmol/g) in DMSO (4 mL). DIEA (? mL) was added dropwise to the solution and the reaction mixture was left to stir at r.t. for 1 h before being heated to 70°C for 2 h. The eluant was filtered off and washed with DMF (3 x 10 mL), CH₂Cl₂:MeOH (1:1, 2 x 10 mL) and CH₂Cl₂ (2 x 10 mL) before being dried. The cyclic peptide 10 was cleaved from resin using HF:p-cresol, 5.5 mL, 10:1, for 1h at 0°C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether before being dissolved in the HPLC buffer and lyophilized. Analytical HPLC (20-70% B over 20 min) showed two peaks; a) linear peptide ES-MS M_r 668.4 (calcd 669.3), and cyclized material ES-MS M_r 650.4 (calcd 650.3).

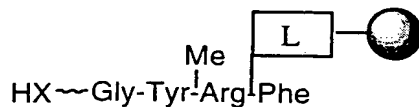
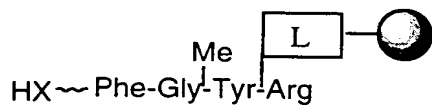
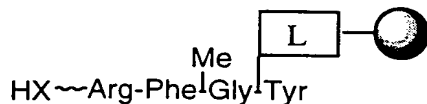
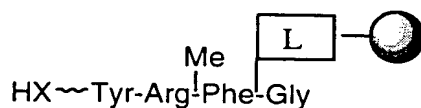
Example 12 Ring contraction, backbone substitution and backbone linker

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Our current backbone linkers can be attached to any atom of the peptide backbone. As the data in Table 3

suggest, more than one N α -substituent results in the best yields of cyclic tetrapeptides for the examples studied. In combination with ring contraction this provides a powerful approach for the synthesis of cyclic peptides.

5 The peptides outlined below are synthesized using this combined approach. These all contain 2 N α -substituents (one is the linker L) and a ring contraction auxiliary. These are cyclised and the purity and yields of products are examined. Reversible N α -substitution in
10 replacement of methylation is also investigated.



HX \sim = ring contraction auxiliary;
X = O, S; L = backbone linker

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments
5 and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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10 following pages, and are incorporated herein by this reference.

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25 September 1998

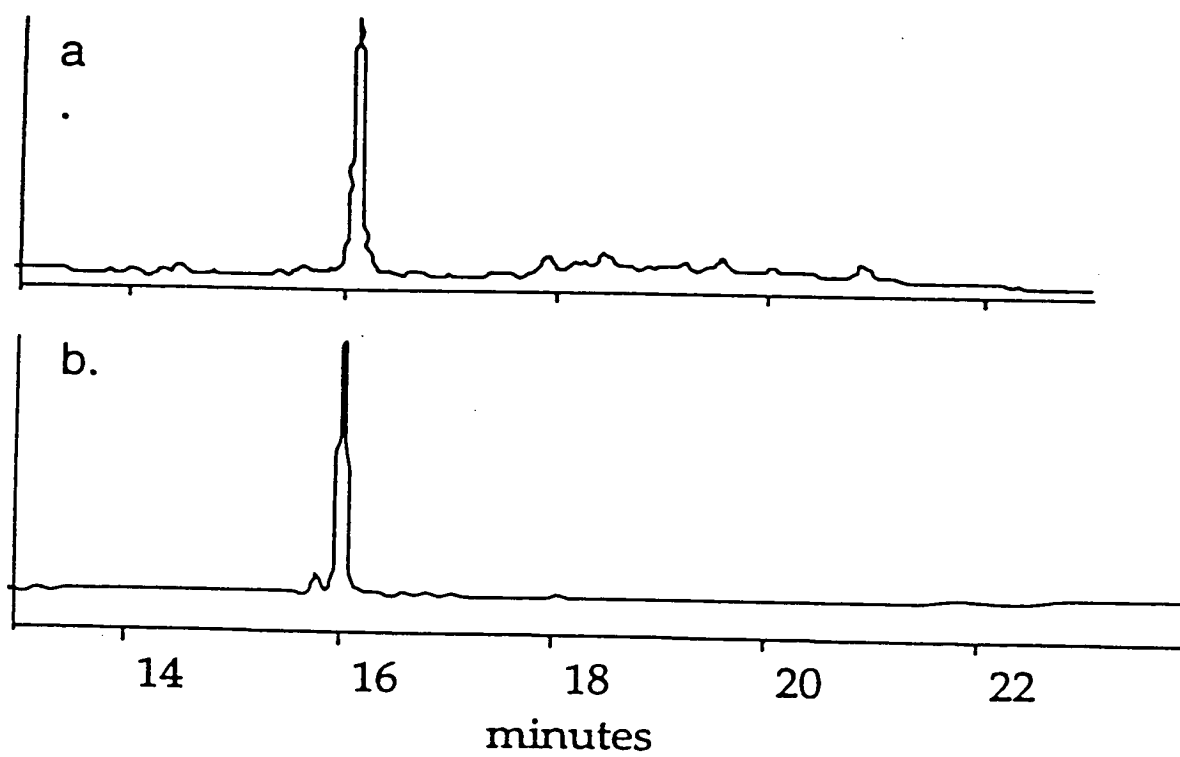


FIGURE 1

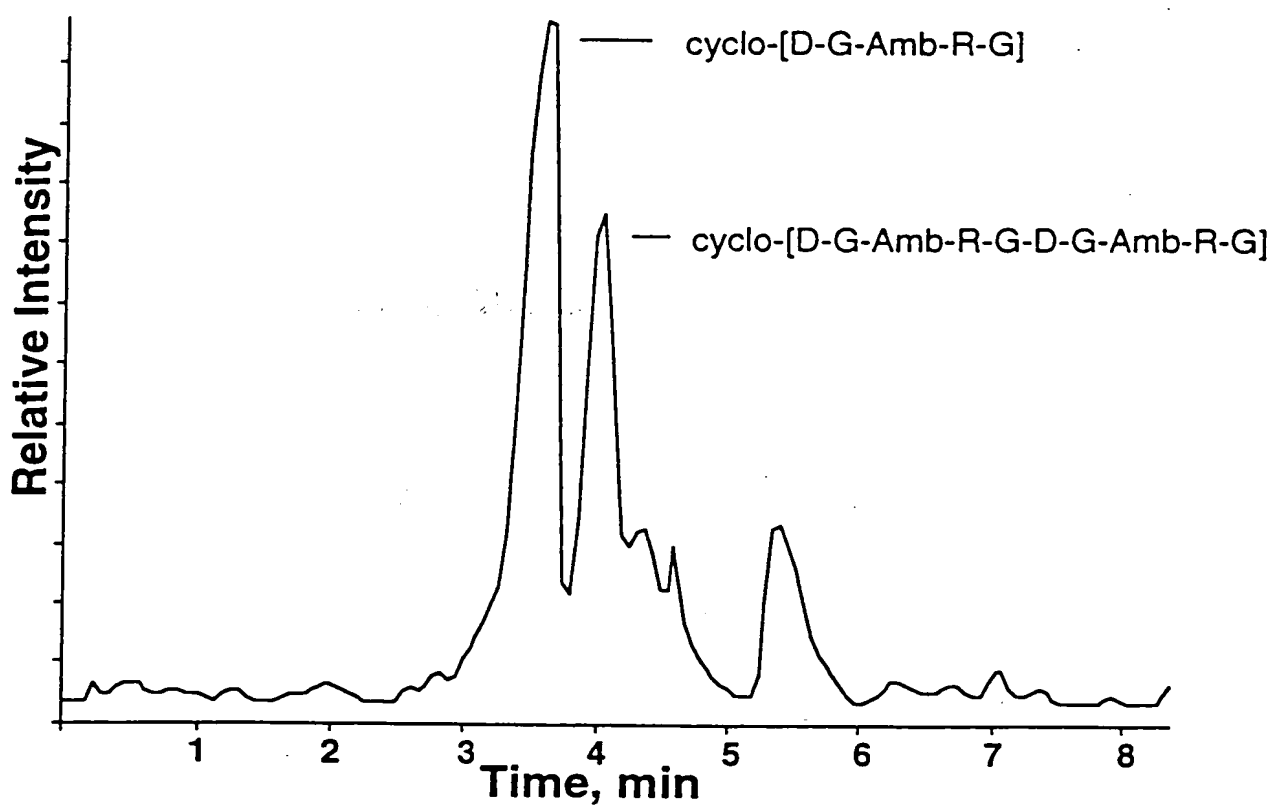


FIGURE 2

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